THE ROLE OF CYTOMEGALOVIRUS IN THE INFLAMMATION ASSOCIATED WITH HIV INFECTION AND AGING

by
Nicholas Rodin

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Abstract

Background

Highly active antiretroviral therapy (HAART) has enabled people with HIV infection (HIV+) to live longer but has also resulted in an increase in aging-related conditions, such as frailty. Chronic inflammation is a hallmark of HAART-treated HIV infection and aging. Cytomegalovirus (CMV) infection can cause chronic inflammation and is prevalent in both HIV-infected and elderly people. CMV-specific T cells comprise 10-20% of the T cell pool, and in a recent study, T cell responses to CMV correlated with inflammatory markers. However, the relationship between CMV levels in peripheral blood mononuclear cells (PBMC) and the magnitude of T cell responses to CMV is unknown.

Objectives

1) To quantify the amount of CMV DNA in PBMC and determine if this amount is related to HIV and frailty status; 2) to determine if this amount is related to T cell responses to CMV and if this relationship differs by HIV and/or frailty status.

Methods

Droplet digital polymerase chain reaction (ddPCR) was used to detect rare CMV DNA in PBMC from men in the Baltimore-Washington DC site of the Multicenter AIDS Cohort (MACS) study with known HIV and frailty status. Numbers of CMV-responsive T cells were compared between donors with and without detectable CMV DNA and among HIV-frailty subgroups.

Results

CMV DNA was detected in PBMC but levels were unrelated to HIV or frailty status. Donors with detectable CMV DNA had higher T cell responses in some cases, and donors without detectable CMV DNA had higher responses in other cases, depending on the HIV-frailty subgroup, the class of T cells (CD4 or CD8), and the specific CMV antigen.

Conclusions

CMV DNA was quantified in PBMC by ddPCR, and specific CMV DNAs that were or were not detected correlated with specific T cell responses to CMV. These responses may help control the amount of CMV DNA, especially responses that were greater when CMV DNA was not detected. Understanding how different CMV strains are related to the T cell response to CMV, as well as knowing who will better control CMV infection, may aid in vaccine development for CMV and in prevention of inflammation-related conditions.

Advisor: Dr. Joseph Margolick

Readers: Dr. Lisa Jacobson, Dr. Alan Scott, Dr. Sean Leng

Alternates: Dr. Jay Bream, Dr. John Nicholas

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I. Introduction

Highly-active antiretroviral therapy (HAART) was introduced in the 1990s to treat people infected with human immunodeficiency virus (HIV), and in the almost-three decades since, HAART has greatly prolonged the life expectancy of these people (1). Today, over 45% of Americans living with HIV infection are over 50 years old (2). Despite being on HAART, which suppresses the HIV to clinically undetectable levels, people living longer with HIV are subjected to age-related chronic conditions known as HIV-associated non-AIDS (HANA) conditions. These HANA conditions include cardiovascular disease and osteoporosis (3), as well as frailty, a clinical syndrome that is associated with chronic inflammation (4). Chronic inflammation is a hallmark of HIV infection, even in people receiving HAART, and is shown by increased serum concentrations of the pro-inflammatory cytokines interferon-gamma (IFNγ) and tumor necrosis factor (TNF), among other markers. The etiology of the chronic inflammatory state present in people with treated, virologically-suppressed HIV infection is unknown, and many factors may contribute to this, as discussed in section II.B. One possible contributing factor is infection with cytomegalovirus (CMV), which causes a persistent viral infection. In support of this possibility, nearly all HIV+ people and elderly people are seropositive for CMV.

Chronic inflammation is also a key feature of aging. Dysregulation of the immune system and increased production of inflammatory cytokines such as TNF and interleukin-1 (IL-1), contribute to aging-related conditions such as frailty (5). Frailty is an important clinical

condition in aging that results from the failure of many physiological systems (6). A consequence of frailty is an inability to respond effectively to stressors that increase the susceptibility to injury and subsequent morbidity and mortality. Frailty is also associated with chronic inflammation, with higher serum concentrations of inflammatory markers in frail people (7). A recent study also linked frailty with CMV infection by identifying correlations between the prevalence of CMV-specific T cells and serum levels of inflammatory markers such as IFNγ, TNF, and IL-6 (8). However, no studies have addressed the relationship between systemic inflammation and the amount of CMV present in the peripheral circulation.

The present study was undertaken to address this gap. Specifically, this project aimed, first, to detect CMV in peripheral blood mononuclear cells (PBMC) from both people with and without (HIV−) HIV infection, and second, to determine the extent to which the presence of CMV correlates with the magnitude of the T cell response to CMV, as shown by the number of T cells producing cytokines in response to stimulation with CMV antigens.

This Introduction provides background on inflammation and its importance in HIV infection (see section I.A). Next, it describes the aging process, focusing on the key role of cell damage and senescence (sections I.B.1 and I.B.2), and the significance of chronic inflammation in aging (section I.B.3). Section I.C discusses frailty and its increased prevalence in people with HIV and aging, and section I.D discusses characteristics of CMV and CMV infection, including its epidemiology (section I.D.1), its structure and genome (section I.D.2), its replication cycle (section I.D.3), and the immune responses to the phases

of CMV infection (sections I.D.4 and I.D.5). Section I.D also summarizes the current understanding of the latent phase of CMV infection and the connections between CMV infection and aging (sections I.D.7 and I.D.8, respectively). Finally, section I.E discusses how the current project built on the previous studies of the role of CMV in the pathogenesis of the chronic inflammation seen in both HIV infection and aging. This Introduction concludes with the specific aims and hypotheses of the current project.

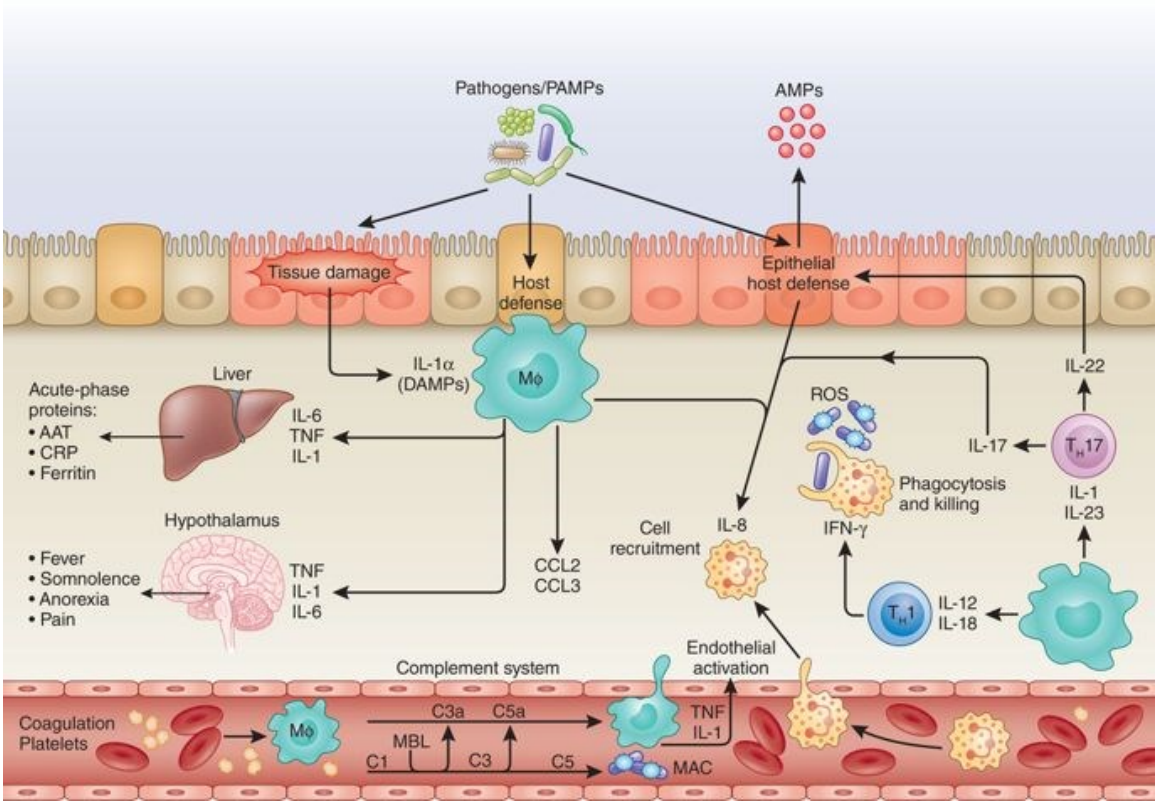
A. Inflammation and Its Role in HIV Infection

1. Inflammation

Inflammation is defined as a "protective response to stimulation either by pathogens or to host cell signals such as cellular damage that results in the clearance of dead or dying cells and initiates tissue repair" (5). It is a significant factor in the pathogenesis of a variety of diseases and conditions, such as atherosclerosis, cancer, autoimmunity, chronic infections, and aging (4). As shown in Figure 1.1, inflammation is stimulated by pattern recognition receptors (PRRs) expressed on myeloid cells and lymphocytes that sense either highly-conserved pathogen-associated molecular patterns (PAMPs) or endogenous damage-associated molecular patterns (DAMPs) (9). Cellular activation through PAMPs or DAMPs results in the production of pro-inflammatory cytokines such as TNF and IL-1β that can recruit and activate macrophages locally or exert systemic effects that elicit the production of C-reactive protein (CRP) by the liver and activate platelets (5). The pro-inflammatory cytokines activate endothelial cells, which results in increased vascular permeability, allowing circulating immune cells to reach the site of infection. However, it can also cause capillary

leakage, vasodilation, and hypotension, which, if not regulated, can be prolonged and can become significant problems associated with chronic inflammation.

Figure 1.1. Overview of the Inflammatory Response (from (5)).
This figure shows the various cells that respond to introduction of a foreign substance, causing inflammation, and shows the molecules produced by those cells. Cells such as macrophages and T cells produce pro-inflammatory molecules that include cytokines and chemokines and have a wide range of effects. For example, the cytokine IFNγ, which is produced by CD4 T cells, activates neutrophils, while IL-22, which is produced by Th17 cells, stimulates antimicrobial peptides (AMPs) such as defensins (10). Chemokines, such as CCL2, recruit immune cells that are required for phagocytosis and for killing invading pathogens.



2. Inflammation in HIV Infection

Chronic inflammation plays an important role in the pathogenesis of HIV infection. HIV infection is associated with an increase in activated T cells (11), inflammatory monocytes, and inflammatory cytokines compared to individuals not infected with HIV (12). Initial HIV

infection is associated with rapid release of a large quantity of pro-inflammatory cytokines, such as IFNα, IFNγ, TNF, IL-6, IL-10, and IL-15 (13). The number of activated T cells also increases dramatically (14). Moreover, monocytes play a key role in the inflammation seen in HIV infection by producing the pro-inflammatory cytokines IL-6 and TNF (15). HIV infection is also associated with a hypercoagulable state, which in combination with the inflammatory environment results in increased morbidity and mortality (15, 16), and the degree of inflammation is prognostic for the progression of HIV infection if it is not treated (17). All of these processes persist through the chronic phases of HIV infection.

Although HAART greatly reduces inflammation, an inflammatory state persists in treated HIV+ individuals (18). People receiving HAART have on average 50% higher serum levels of IL-6 than demographically similar people not infected with HIV (15), and IL-6 levels are associated with increased risk of mortality and aging-related chronic diseases (16). Markers of monocyte activation such as soluble CD14 (sCD14), derived from circulating monocytes, are elevated in HIV-infected people receiving HAART, and are predictive of morbidity (19).

This heightened inflammation and T cell activation could be a result of continuous HIV replication (e.g., at very low levels even in virologically-suppressed people receiving HAART) (20). It could also be due to infections with pathogens other than HIV, such as CMV, which cause inflammation by activating T cells.

B. Aging

Chronic inflammation is also commonly seen in aging (21). Aging involves the gradual accumulation of cellular damage over time. This accumulation can lead to dysregulation and subsequent decline of multiple systems throughout the body, including the immune system. The dysregulation of the immune system causes cellular hyperactivity, which in turn can cause further inflammation.

1. Cell Damage in Aging

One of the most common markers of aging is the accumulation of genetic damage (22). The continual threat of DNA damage comes from exogenous factors (e.g., pathogens and chemicals) as well as endogenous factors (e.g., errors in DNA replication). The DNA damage that can arise includes point mutations, translocations, chromosomal gains or losses, and telomere shortening. These DNA alterations may modify the expression of genes and transcriptional pathways, resulting in cellular dysregulation. Dysfunctional cells are normally removed by apoptosis or senescence, and failure to do so may impact other cells or tissue function with numerous significant consequences (e.g., inflammation).

Telomeres, are structures located at the terminal ends of DNA chromosomes which protect the chromosomal DNA from the inability of DNA polymerase to effectively replicate the DNA at the ends of the chromosome. The inability of telomeres to fully replicate and their gradual shortening over time, makes them particularly susceptible to damage during aging.

DNA damage at telomeres induces senescence and/or apoptosis, a function of the normal aging process.

2. Cellular Senescence in Aging

Along with genetic and cell damage, cellular senescence occurs during the normal aging process. Cellular senescence is the stable arrest of the cell cycle coupled with phenotypic changes, such as resistance to apoptosis and various alterations in gene expression (23). Senescence can be triggered by telomere shortening (as discussed in section I. B.1), as well as by other types of DNA damage, along with de-repression of the INK4/ARF locus and dysregulation of other signaling pathways, all of which occur during aging (24). Senescent cells must be removed and replaced by progenitor cells to maintain the cell number and prevent system dysfunction. The increased number of senescent cells in the elderly may arise due to an inability to remove these cells, aggravating any damage caused during the aging process and contributing to inflammation.

3. The Significance of Inflammation in Aging

Senescent cells can alter their secretomes to produce large amounts of pro-inflammatory cytokines. The chronic inflammation seen in aging, known as "inflammaging" (25), may arise from an accumulation of pro-inflammatory tissue damage. This is the result of the immune system becoming more dysfunctional and less effective at clearing pathogens and an increased number of senescent cells that can secrete pro-inflammatory cytokines (21) DAMPS, such as extracellular adenosine triphosphate (ATP) and reactive oxygen species (ROS), which are elevated during aging (26) and in senescent cells (27), activate the NOD-like receptor protein 3 (NLRP3) inflammasome. The NLRP3 triggers the increased

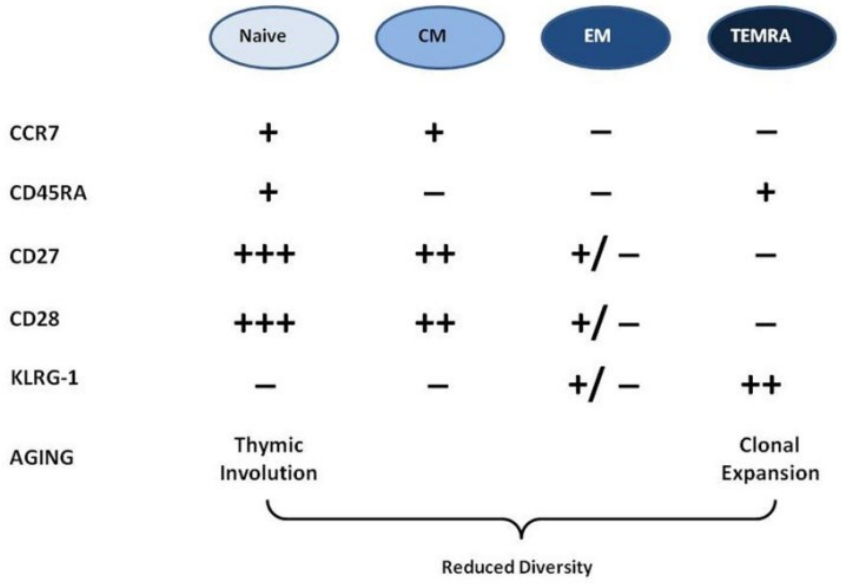
production of IL-1β, TNF, and interferons (28). A result of the increased production of pro-inflammatory cytokines is defective inflammatory responses. Conditions related to aging such as obesity, type 2 diabetes (29), and atherosclerosis (30), all exhibit defective inflammatory responses that contribute to their pathogenesis. In addition, inflammation inhibits stem cell function, impairing the replacement of dead or damaged cells and exacerbating the damage done by the inflammatory environment (31).

"Inflammaging," together with a decline in the adaptive immune response, prevents the immune system from effectively clearing infectious pathogens and infected cells. Increased activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and the NF-κB pathway have been identified as key markers of aging (32). Inflammation (TNF, IL-1, and IL-6) activates NF-κB in the hypothalamus, which causes a reduction in gonadotropin-releasing hormone (GNRH) (33) (Figure 1.1). Loss of GNRH is associated with many aging-related conditions such as bone fragility, muscle weakness, and skin atrophy (33).

The most significant changes to the immune system during aging occur in the adaptive immune system. Weakening the immune system leads to an increased susceptibility to infections, cancers, autoimmune disease, and chronic inflammatory disease. Aging is associated with an increase in memory cells and a decrease in naïve T cells, especially in CD8 T cells but also in CD4 T cells (34). Memory cells protect the host from re-infection with a given pathogen, with the survival of memory cells driven by IL-7 or IL-15 (35). As shown in Figure 1.2 (34), in elderly people, repeated exposure to pathogens results in a higher number

of differentiated T-effector memory (T_{EM}) cells and a higher number of terminally-differentiated memory cells re-expressing CD45RA (TEMRA). The decrease in naïve T cells due to thymic involution and exposure to specific antigens, along with the increased number of clonally-expanded memory cells, results in an unbalanced naïve/memory cell ratio (36). There is a strong correlation between age and the total number of TEMRA CD8+ T cells (37). However, many of these TEMRA are dysfunctional. The inability of TEMRA to produce cytokines in response to stimulation, as well as their inability to proliferate, results in memory cells that may be senescent (34). As discussed in section I.B.2, senescent cells are not just dysfunctional but can also trigger inflammatory responses that inflict significant, widespread damage.

Figure 1.2. Phenotypic Characterization of T Cell Changes in Aging (from (34)). During the aging process, there is reduced diversity among the T cell population due to thymic involution and clonal expansion. This figure shows the various markers expressed on T cell populations at different stages of differentiation and more highly expressed in aging: Naïve T cells, central memory (CM), effector memory (EM), and terminally differentiated T-effector memory expressing CD45RA (TEMRA) cells.



C. Frailty

1. Characteristics of Frailty and the Frailty Phenotype

Frailty is an important clinical syndrome characterized by a loss of physiological reserves and an increased vulnerability to injury, and subsequent morbidity and mortality (38). In addition, frailty results in an inability to respond effectively to daily or acute stressors and triggers a downward spiral towards functional decline (38). Frailty can occur not only with aging, but also as a result of a variety of illnesses (e.g., cancer and congestive heart failure) or even without any specific disease present. Frailty has proven difficult to define, but a widely-used frailty phenotype (FP), known as the Fried FP, is defined as the presence of three or more of the following conditions: weakness as measured by grip strength, low physical activity, slow motor performance measured by walking speed, exhaustion, and unintentional weight loss (39). Using the Fried FP, the estimated prevalence of frailty in the United States is about 10% of men and women age 65 and older (39). The Fried FP predicts the onset of serious conditions such as acute illness, cognitive decline, disability, and mortality (39). It is also an important risk marker for the development of cardiovascular and renal diseases as well as some cancers (39). Moreover, frailty can be a marker for immune decline in the elderly and has been used as a marker to identify people who will not respond well to the influenza vaccine (40) putting them at higher risk of contracting seasonal influenza.

2. The Role of Inflammation in Frailty

Frailty and chronic inflammation both involve the deterioration and dysregulation of multiple physiological systems, and chronic inflammation is one of the possible causes of frailty (38). Frailty is associated with elevated serum concentrations of inflammatory markers such as CRP, IL-6, and TNF, which contribute to the pro-inflammatory phenotype and are also associated with increased morbidity and mortality (41). CD8+ CD28- T cells and CCR5+ T cells that can produce type-1 pro-inflammatory cytokines are also elevated in people with frailty (42). The potent pro-inflammatory cytokine CXCL-10 is constitutively expressed on monocytes and correlated with higher IL-6 levels in frailty (43). Together, the elevated levels of these inflammatory markers suggest that inflammation plays a key role in the pathogenesis of frailty.

3. Frailty in People with HIV Infection

In HIV+ men who have sex with men (MSM), the frailty-related phenotype (FRP) described by Desquilbet *et al.*, which uses the same criteria as the Fried FP but without the grip strength criterion, serves as a valid measure of frailty in HIV+ men (44). The FRP was shown to have a lower prevalence in people receiving HAART than in those not receiving HAART (44). Additionally, the presence of the FRP when starting HAART was a good predictor of overall and AIDS-free survival (44). In 2007, the MACS began assessing all five components of the Fried FP at each study visit. In one study, the prevalence of the FP was 12% in HIV+ and 9% in HIV− men, and this prevalence increased with age regardless of HIV status but was significantly greater in HIV+ compared with HIV− men aged 50-64

(45). That study went on to show that among FP+ men, 34% HIV+ and 38% HIV− had two or more comorbidities (45).

Frailty in virologically suppressed HIV+ people displays inflammation above the levels seen in HIV-infected people on HAART. A study of 24 serologic markers for T cell, B-cell, or monocyte activation, was performed on serum stored at each Study to Help the AIDS Research Effort (SHARE) study visit (7). A multiplex assay to detect various serologic cytokines (IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, GM-CSF, IFN-γ, and TNF) and chemokines (CCL2 (MCP-1), CCL4 (MIP-1β), CCL11 (eotaxin), CCL17 (TARC), CXCL8 (IL-8), and CXCL10 (IP-10) was used. The results were that frailty was significantly correlated ($p<0.002$) with immune activation markers IL-6, soluble CD14, and soluble TNF receptor 2 (TNFR2), in frail HIV+ men compared to non-frail HIV+ men (7). CRP levels were almost 50% higher levels in HIV+ frailty+ men ($p<0.002$) than in HIV+ frailty− men, even after adjustments were made to factor donor characteristics (such as BMI and smoking), and comorbidities. Most of these elevated markers are produced by activated monocytes or macrophages, suggesting that these cells play a role in the inflammation seen in HIV+ frail men.

Another study found that frailty was associated with increased levels of activated CD8 T cells (expressing CD38 and HLA-DR) and serum IL-6 in HIV+ people (46). In another study, frailty was significantly associated with higher IL-6 and soluble TNF receptor 1 (TNFR1) levels (47). Finally, in a third study a strong correlation was found between the CD4+ IL-2 response to CMV peptides and serum IL-6 in HIV− non-frail people, and this response was predictive of the development of frailty (8). Specifically, HIV− nonfrail

donors in the highest tertile of CD4 IL-2 responses to CMV developed frailty at a significantly faster rate than those in the lower 2 tertiles; this relationship, however, was not found for HIV+ nonfrail donors. This study is discussed in greater detail in section I.E.

D. CMV Infection

This project focused on the role of CMV infection in inflammation because, nearly all elderly adults are CMV seropositive (CMV+), and 90% or more of HIV+ people are also CMV+. There is also a huge T cell response to CMV (48), and this response had recently been shown to correlate with systemic inflammation as measured by serologic and cellular markers of immune activation (7). As discussed earlier in the chapter, inflammation is common to both HIV infection and the normal aging process. CMV causes chronic inflammation, making it an excellent candidate for causing, or at least contributing to, the inflammation seen in HIV infection and aging. This section provides background information on CMV, its replication cycle and gene expression, the immune response to it, including inflammation, and finally the relationship between CMV, HIV infection, and aging.

1. Epidemiology of CMV Infection

Although CMV is found around the world and its prevalence increases with age, the distribution of CMV varies across the world. In developing countries, CMV infection is acquired earlier in life, in contrast with the United States and Europe, where it is more prevalent among lower socioeconomic groups, especially in immigrant communities from developing countries (49). CMV infection is spread by exposure to infected bodily fluids, with mucosal epithelium the most common site for primary infections. CMV can also be

transmitted from a mother to her fetus during pregnancy, by crossing the placenta. Congenital infections can be very severe and can cause life-threatening cytomegalic inclusion disease (CID) (50). The risk to fetuses is significant because CMV prevalence is slightly higher in women of childbearing age than in men of a similar age (51).

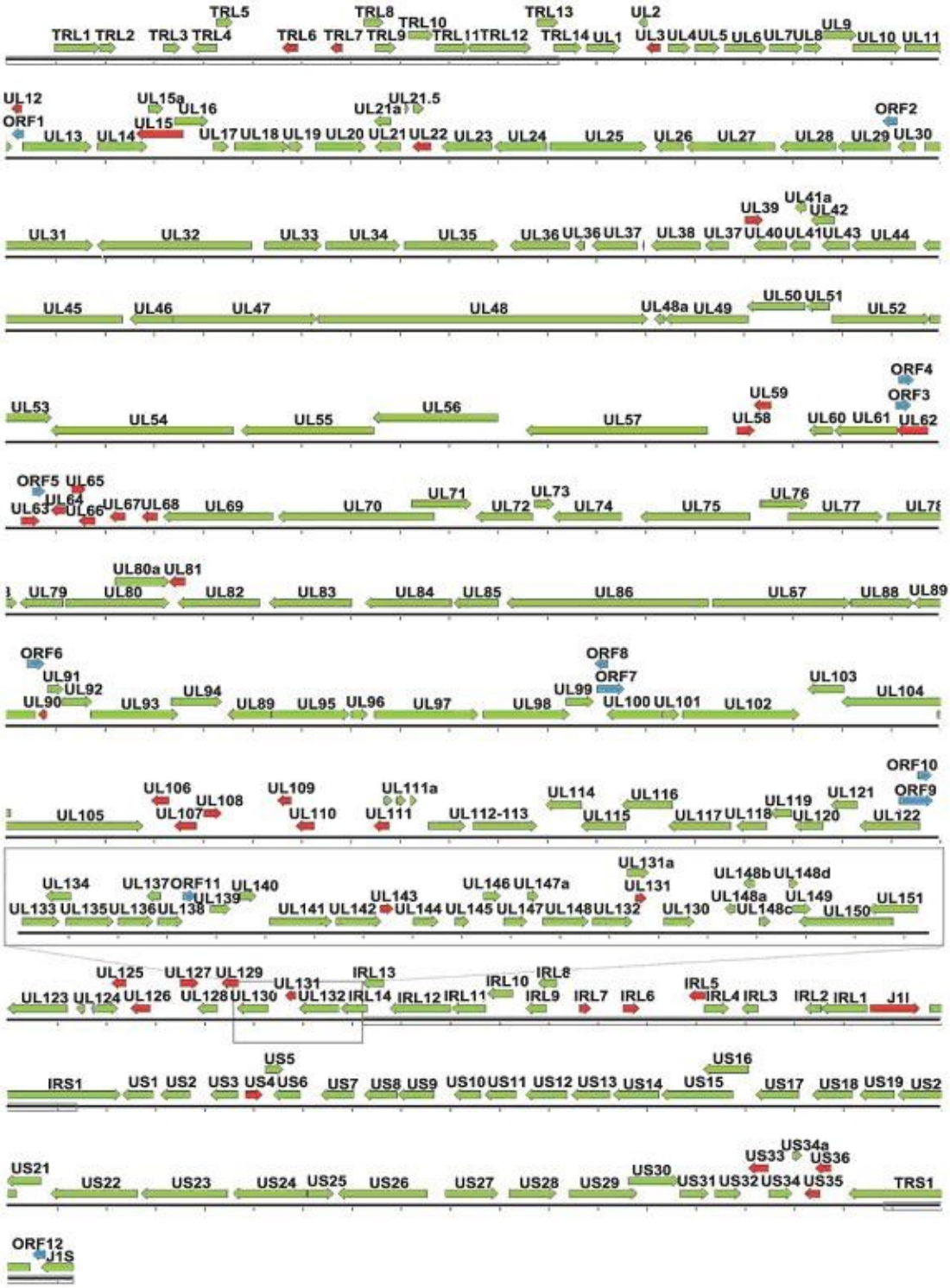
2. Genome and Virion Structure of CMV

CMV is the largest virus that causes human disease. It is a member of the herpesvirus family and has a DNA genome 230kb long with over 750 identified protein-encoding open reading frames (ORFs) (52), as shown in Figure 1.3. The CMV genome is composed of unique long (UL) and unique short (US) regions that are flanked by terminal and internal repeat sequences. These repeat sequences contain genome cleavage and packaging signals that allow isomerization of the viral genome during replication (53).

The CMV virion particle is 230nm in diameter (54) with a structure that is characteristic of other herpesviruses. It has a DNA core inside a highly-stable 130nm icosahedral capsid, which is larger than that of other herpesviruses to accommodate CMV's large genome. The capsid is surrounded by an envelope, which contains viral glycoproteins to control attachment and entry into cells (50). The capsid is composed of four core proteins that are essential for CMV replication (55). The DNA genome is contained inside the nucleocapsid along with two virion RNAs (50). The tegument, composed of 32 virus-encoded phosphorylated proteins, encloses the nucleocapsid. The tegument is surrounded by a lipid bilayer envelope originally derived from the endoplasmic reticulum (ER)-Golgi intermediate compartment of the host cell, which is modified by the insertion of virus-encoded

glycoproteins (50). The envelope glycoproteins gB, gH, gL, gM, and gN have essential functions in the replication of viral DNA and are the targets for neutralizing antibodies (gB will be discussed in detail in section I.D.3) (50). Virion proteins also play an important role in cell entry, egress, and cell tropism.

Figure 1.3. The Genomic Arrangement of CMV (from (56)).
The key ORFs in the 230kb genome (56). The green arrows represent ORFs, red arrows designate ORFs unlikely to encode a polypeptide. UL= Unique long sequences. US= Unique Short sequences. Rectangles superimposed on the line represent the sequence-identify terminal repeats. Each mark on the sequence line represents 1,000 bp.



3. The Replication Cycle of CMV

As a result of its large genome, CMV has a replication cycle that takes 48 to 72 hours from viral entry to the final stages of maturation and progeny release (50). The first genes to be expressed (within minutes after infection of permissive cells) are immediate-early (IE) genes. These are followed by delayed early (DE) and then late (L) genes, expressed 24 to 36 hours post-infection. Figure 1.4 shows the key viral-host interactions that determine whether the virus will enter a lytic or latent phase of replication (57). The sequential expression of the IE, DE, and L genes is highly regulated (58). Figure 1.5 depicts the key viral proteins involved in the various stages of the CMV replication cycle (50).

Figure 1.4. Key Viral-Host Interactions to Determine Either Viral Lytic (Productive) Replication or Latency (from (57)).
Signaling pathways are critical for establishing cellular environments for replication or latency. US28, a viral chemokine receptor homolog, functions in both lytic and latent phases. US28 activates IL-6/JAK/STAT3 signaling. IE1 is essential for lytic replication. Cells infected with latent CMV upregulate PI3K or MAPK/ERK activation, leading to upregulation of the protein MCL-1 that inhibits apoptosis and promotes cell survival (59).

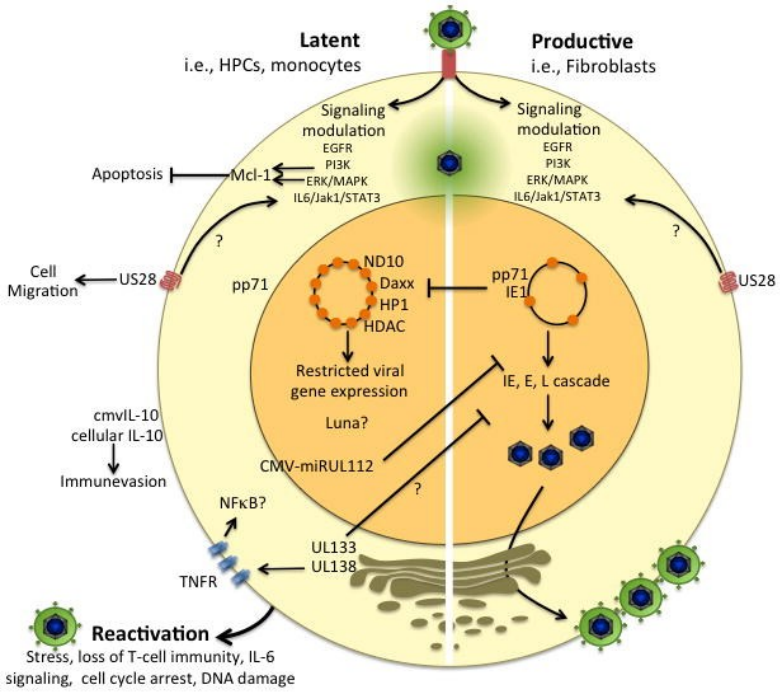
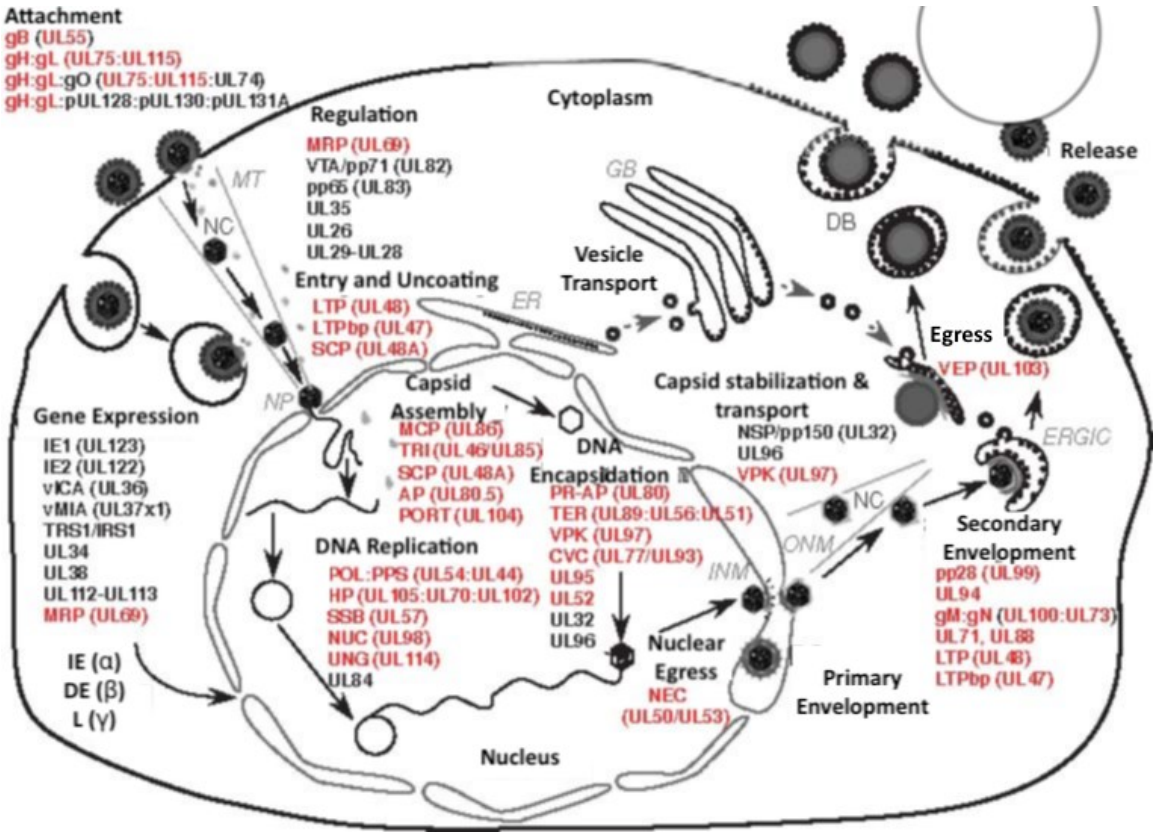


Figure 1.5. CMV Replication Cycle (from (50)).
Key viral proteins involved in the CMV replication cycle are shown, including gB, which is important for attachment, and IE1, which is essential for viral replication.



i. Immediate Early Gene Expression

IE gene expression is controlled by the major IE promoter (MIEP) that activates transcription of IE1 (p72) and IE2 (p86). The MIEP regulates transcription by initiating viral gene transcription immediately after viral entry into permissive cells and by repressing transcription during latency (60) (see section I.D.6 for more on latent CMV). Chromatin remodeling by histone acetyl transferases (HATs) and demethylases positively regulate MIEP expression. Conversely, chromatin remodeling by histone deacetylases (HDACs) and

methyltransferases negatively regulate MIEP expression (61). Tegument proteins pp71 relieve initial HDAC-mediated repression of the MIEP, allowing the initiation of IE gene transcription (62). By suppressing HDACs, MIEP proteins IE1 and IE2 play an active role in chromatin remodeling and continuing gene expression, and viral replication (60). During latency (as discussed in section I.D.7), the MIEP region remains unmethylated due to CPG suppression, preventing IE transcription and viral replication (63).

IE1 and IE2 result from alternative mRNA splicing of major immediate early genes (MIE), UL123 and UL122, respectively (64). IE1 and IE2 are responsible for initiating productive viral genome replication by regulating the MIEP, and also suppresses the immune response, promoting the survival of the virus inside the cell. IE1 associates with and disrupts nuclear domain 10 (ND10) (also known as promyelocytic leukemia protein (PML) bodies) (65), inhibiting HDACs that also interact with ND10 and enabling MIEP gene expression as well as DE and L gene transcription (66). IE1-deficient viruses are unable to replicate efficiently due to the inability to inactivate HDACs and activate DE gene expression (61). These replicative-deficient viruses are still able to disrupt ND10, suppress interferon response, and initiate DNA replication (67).

ii. Delayed Early and Late Gene Expression

DE gene expression peaks between 8 to 12 hours post-infection but continues through to 24-hours post infection (h.p.i) (68). DE genes are vital for viral DNA synthesis, and their loss significantly impairs viral replication (69). The expression of UL54, a CMV-encoded DNA polymerase, starts around 8 h.p.i. And remains constant throughout the L phase (50).

Other DE genes regulate the expression of other viral genes, which is important for efficient viral replication.

The L phase of replication is considered to begin 24 h.p.i. L gene proteins control the final stages of viral maturation, including capsid maturation, DNA encapsidation, and the exit of the virion from the cell (70). One of the key genes expressed during the L phase is UL55-coded glycoprotein B (gB), a highly-conserved envelope protein that is important for attachment and entry and is a major target for neutralizing antibodies (50). It forms a trimer on the envelope surface that enables fusion and attachment with the cell membrane, allowing entry into the cell. Cellular receptors for gB are unknown but may include cell surface integrins such as α2β1, α6β1, and αvβ3 (71) that are present on all cells, and the epidermal growth factor receptor (EGFR) on monocytes (72). As the virion matures, gB is cleaved to create a 116 kd surface component that is bound to a 55 kd transmembrane component (50). Antibodies to these domains can prevent viral binding and entry into cells, and soluble gB has been shown in human trials to elicit protective immunity (73).

4. The Immune Response to Primary CMV Infection

Primary CMV infection in peripheral tissues triggers a strong innate immune response with activation of natural killer (NK) cells and then an adaptive response that includes a diverse antibody and T_{EM} response (74). NK cells play a key role in controlling and fighting CMV infection by killing CMV-infected cells and by producing type I IFN, as illustrated by the rare people who lack NK cells and are extremely susceptible to multiple herpesvirus infections (75). The important role of NK cells in fighting CMV is further highlighted by

the significant increase in CD56dim NK cells in the blood of healthy CMV-infected people over 60 years old (76). NK cells also produce IL-22, which recruits neutrophils that contribute to control of the virus (77). Initial IFN production by splenic stromal cells occurs 10- to 12 h.p.i and relies on signaling by lymphotoxin αβ, which is produced by B-cells (78). IFN is subsequently produced by both plasmacytoid dendritic cells (DC) and conventional DC, which also produce high levels of IL-12 and IL-18, which together activate NK cells and prime the adaptive immune response (79). In addition to NK cells, mast cells activated within hours of infection produce chemokines that recruit CD8+ T cells to the site of infection (80).

5. The Immune Response to Persistent CMV Infection and Chronic Inflammation

Once the initial infection is controlled, primarily by the innate immune system, CMV infection enters a persistent phase within infected tissue, where it may persist for months or years. Here, the adaptive immune response plays an important suppressive role. The expansion of effector T cells is sufficient to control the primary CMV infection in peripheral tissues. A broad CD4 and CD8 T cell response accounts for up to 10-20%, or even more, of all circulating T cells in CMV-infected individuals, and it is this response that will eventually contract to generate a stable memory pool (48). Sylwester *et al.* tested the T cell responses to many thousands of CMV peptides covering more than 150 ORFs, and demonstrated that there are specific T cell responses to most or all of them (48). The breadth of the T cell response to CMV has also been shown in both HIV− (48) and HIV+ (81) people.

Such a large expansion of CMV-specific T cells leads to a net reduction in the diversity of the overall T cell pool, which compromises immune function (82).

A subset of the primed T_{EM} cells, comprised of "inflationary memory" cells, does not contract but rather can expand due to the persistence of viral antigens (82). The expansion of CMV peptide-specific T_{EM} cells can be very high in some people, with over 20% specific for a single antigen in some cases (83). Inflationary memory cells often lack one or both of the co-receptors CD27 and CD28 (84) and express high levels of NK cell receptors, such as CD57 and KLRG1, along with the effector molecules perforin and granzyme B (85). This T_{EM} subset is maintained by a small population of CD27hi CD8 central memory T cells and is dependent on the initial dose of viral inoculum (86). These inflationary memory cells also do not show typical signs of cell exhaustion, despite their continued exposure to CMV antigens.

The persistence of inflationary memory cells may be due to expression on CMV-specific CD8+ T cells of molecules normally associated with NK cells, such as CD56 and killer-cell immunoglobulin-like receptors (KIRs) (82). CD45RA, more often expressed on naïve T cells than memory cells, is also more highly expressed on these CMV-specific CD8 T cells, and may also contribute to the long-term survival of these cells (87). The high frequency of oligoclonal CMV-specific CD8+ T cells indicates that extensive clonal expansion and selection have occurred, often with only a few epitope-specific clones dominating (88). Cells that have reverted to CD45RA expression have high-avidity T cell receptors (TCRs) (89), which means they will bind more strongly with antigens and enable the cells to be more

likely to survive. The survival of these T cells causes clonal diversity to decrease over time, leading to an increase in high-avidity CMV-specific CD8 T cell clones (90).

As CMV-specific CD8+ T cells expand in CMV+ people, the number of CMV-specific CD4+ cells also increase with age with a more terminally-differentiated phenotype (91). The priming of CMV-specific CD8+ T cells requires CD28-mediated co-stimulation, but CD28 is not essential for memory inflation (82). CD27 binding to CD70 (92), as well as the co-stimulatory molecule OX40 (93), are important for both CD8 priming and also for memory expansion. IL-2 helps the differentiation of effector T cells during the priming process. IL-2 may be produced by CD4 or CD8 T cells and in addition to antigenic stimulation is required for CD8 memory cell inflation (82).

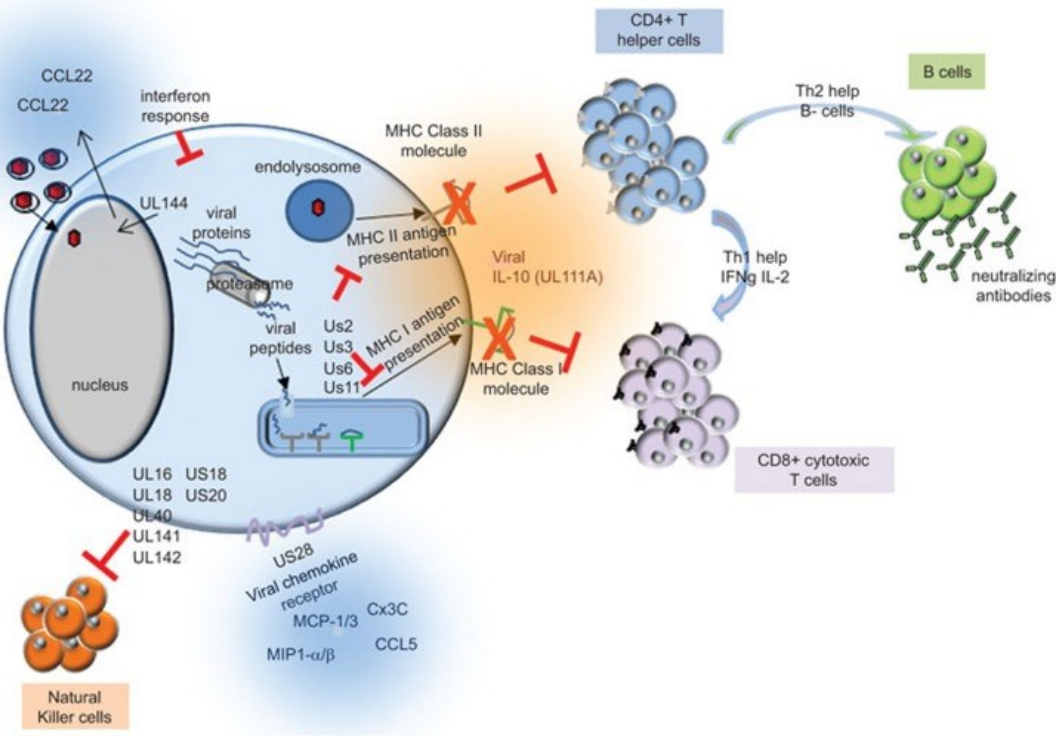
CMV also induces a strong antigen-specific antibody response. Immunoglobulin (Ig) G levels rise throughout CMV infection during the persistent stages and seem to plateau as the virus is controlled (74). IgM levels drop significantly after the initial primary infection is cleared (94). Despite the strong antibody response to CMV by B cells, immune control of CMV is no different in mice lacking B cells (95). This indicates that T cell responses are more important for control of human CMV infection that antibody levels.

6. Mechanisms Used by CMV to Evade the Host Immune Response

CMV modulation of the host immune response includes altering pathogen receptor signaling, inhibiting cytokine and interferon activation, and preventing cell death to ensure

that virions mature within the infected cell (as shown in Figure 1.6). NF-κB activation occurs immediately after exposure to viral particles and again 24-hours post infection, in response to factors such as IFN and TNF, which are a result of the primary response. Cytosolic DNA censors (ZBP1) activate interferon regulatory factor-3 (IRF3) (96) and NF-κB (97) resulting in pro-inflammatory cytokine (IFNγ and TNF) production. Viral proteins made during infection can limit the impact of the IFN response on host cells. For example, IE1 forms a complex with STAT1 and STAT2 to prevent its binding to IRF9, which results in decreased activation of IFN-responsive promoters (98).

Figure 1.6. CMV and Immune Interactions During Lytic Infection (from (99).
CMV viral proteins mediate evasion of the host immune response. This evasion includes inhibiting the IFN response and preventing T- and NK cell activation and recognition and subsequent killing of CMV-infected cells. Viral IL-10 encoded by the CMV also suppresses immune activation.



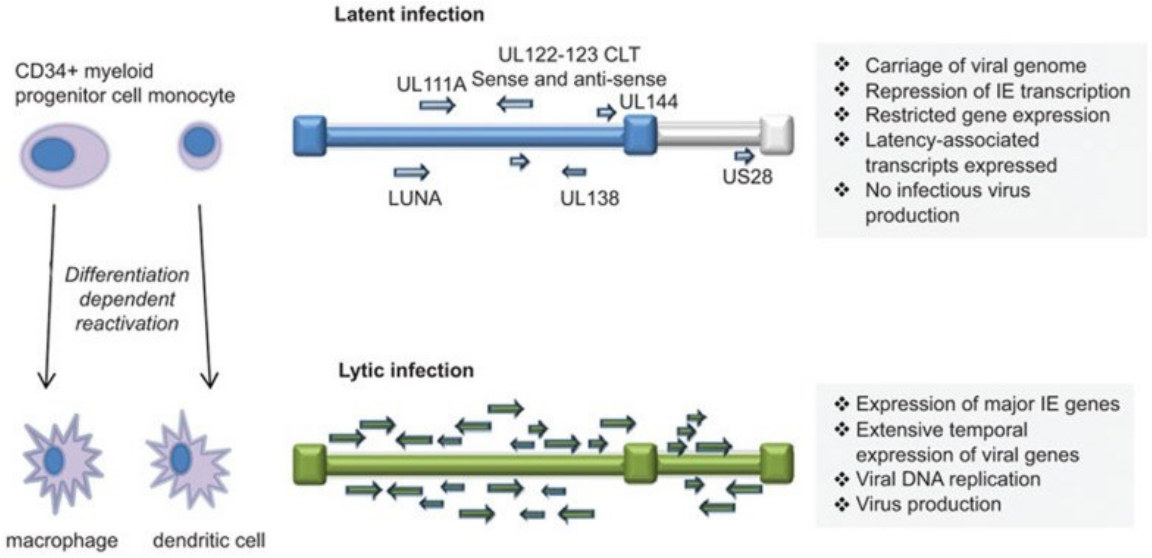
7. The Latent Phase of CMV Infection

Latent CMV persists in specific cells and tissues, where it exhibits restricted viral gene expression and no viral production. Latent virus has been detected in CD34+ myeloid progenitor cells that reside in the bone marrow, which provide a reservoir of virus that is not eliminated by the host (100). These progenitor cells can differentiate into macrophages and dendritic cells where the virus subsequently can be reactivated. Presence of CMV in these long-lasting cells and progenitors, which can differentiate into macrophages, enables the virus to reside in all tissues throughout the host. Latently-infected cells are rarely detected in the peripheral blood (101). Studies have suggested that, during latency, the frequency of viral genome-positive cells in the peripheral blood ranges from 10^{-4} to 10^{-5} PBMC, with 2 to 15 CMV genome copies per cell (101). During latency the viral genome is maintained in an episomal form in the nucleus.

Most CMV proteins are suppressed during latency (as shown in Figure 1.7), but those that are expressed have an important role in maintaining viral latency and preventing recognition by T cells. Latent proteins LUNA (102), UL138 (103), US28 (104) and vil-10 (105) are the only proteins that have been detected during latent infection. They can be recognized by CD4+ T cells that secrete immunomodulatory cytokines IL-10 and transforming growth factor-beta (TGFβ), rather than IFNγ (106). The importance of IL-10 in the maintenance of latent CMV is highlighted by the CMV-encoded vil-10 homolog that is expressed during latent infection (107). CMV-encoded IL-10 suppresses the immune system by downregulating major histocompatibility complex (MHC) class I and II (108) and preventing DC maturation and function (109). IE1 expression is repressed by the MIEP but may be

reactivated by transcriptional activators such as NF-ϰB or by differentiation and activation of the infected myeloid cells by pro-inflammatory cytokines (110). Cells latently infected with CMV can avoid apoptosis by upregulating the cellular anti-apoptotic protein MCL-1 and by dysregulating the ERK-MAPK signaling pathway in CD34+ cells (111).

Figure 1.7. CMV Replication During Lytic and Latent Infection (from (99)).
In latently-infected cells there is targeted suppression of lytic viral gene expression and expression of a few latently-associated genes detectable, including LUNA, UL138, and US28.



8. CMV in Aging

The interaction between CMV and the immune response during aging may result in an inability to control replication of the virus. This was suggested by a greater amount of CMV DNA in the urine of older people as compared to that of young people, and by the reported increase of latent CMV genomes in CD14+ monocytes in people over 70 years old (112), although these findings have yet to be independently corroborated. High levels of CMV-specific IgG are associated with aging, increased mortality, and cardiovascular disease (49).

Latent CMV is associated with the accumulation of a large number of TEMRA CD8+ CD27- CD28- CCR7- CD62L- CD57+ CD45RA+ T cells with reduced functionality and proliferative capacity (113). The accumulation of CMV-specific CD8+ TEMRA may be due to chronic antigenic stimulation by the latent virus (34). Latency may involve intermittent gene activation that results in incomplete reactivations, causing intermittent antigenic activity that can stimulate clonal expansion of CMV-specific T cells. CMV has been shown to be associated with HIV infection and aging and with the chronic inflammation present in people suffering from these conditions (8). The exact role that CMV plays in the pathogenesis of the inflammatory response seen in HIV infection and aging is an important question that remains to be answered.

E. **Objectives of this Project**

The increasing number of people living longer with HIV since the introduction of HAART (1) has led to a rise in HANA conditions, such as frailty. Frailty has been shown to be a good predictor for the development of serious chronic conditions, such as acute illness, cognitive decline, chronic inflammation, and mortality (39). Chronic inflammation can cause cell and tissue damage and immune dysregulation and is commonly seen in people who are HIV+ and frail. Inflammatory markers, such as TNF, IL-6, and CRP, are elevated in people who are both HIV+ and frail (7).

Persistent viral infections such as CMV can also cause chronic inflammation. Nearly all HIV+ individuals are also co-infected with CMV, and CMV has been associated with a higher risk of frailty and mortality in older women (114). CMV seroprevalence increases

with age and also distorts the immune response. By considerably altering the T cell repertoire, up to 20% (and in some cases higher) of CD4+ and CD8+ T cells are CMV-specific, and the resultant net-shrinking of the T cell pool can compromise immune function.

Whether CMV is responsible for the chronic inflammation seen in HIV+ individuals and in frail people is an important question that has not been answered. Inflammation in aging is a problem that affects people who are both HIV− and HIV+. As more people live longer with HIV infection, understanding the aging process and associated conditions, such as frailty, will become even more critical. Improving our understanding of the role CMV plays in chronic inflammation may help identify new possibilities for alleviating chronic inflammation. This includes people with HIV infection who are at risk of developing frailty and other manifestations of the normal aging process, such as atherosclerosis.

As discussed earlier in this chapter, previous studies have shown that people with HIV infection have a high CMV seroprevalence, which is associated with a higher risk of frailty; they also have very strong T cell responses to CMV peptides (115), and higher prevalence of frailty at earlier ages than HIV− people (45). However, the role CMV infection plays in the chronic inflammation seen in aging in both HIV+ and HIV− people is poorly understood.

A recent study by Margolick *et al.* evaluated the relationship between T cell responses to CMV peptides, systemic inflammatory markers, and frailty in HIV-infected and -uninfected

men from the Multicenter AIDS Cohort Study (MACS) (8). MACS is an ongoing longitudinal study of HIV infection in MSM, where participants provide blood samples on a semi-annual basis (116, 117). At each visit, HIV status, health indicators, and since 1997 frailty status for the Fried FP are recorded for each participant (more details on the MACS can be found in the Methods). Most HIV+ participants have received HAART and have suppressed their HIV viremia to undetectable levels, greatly reducing or eliminating the effects of HIV viremia. Using CMV peptide pools that covered 19 CMV ORFs (UL28, UL32, UL36, UL48, UL55, UL82, UL83, UL86, UL94, UL99, UL103, UL122, UL123, UL151, UL153, US3, US24, US29, and US32), the number of CD4 and CD8 T cells that produced pro-inflammatory cytokines in response to CMV antigens was measured in 42 MSM. The numbers of responding cells were analyzed by HIV and frailty status, first for the study group as a whole (n=42), followed by subgroups stratified by HIV status (20 HIV−, 22 HIV+), frailty status (21 non-frail, 21 frail), and finally by both HIV and frailty status: HIV−/frailty− (n=10), HIV+/frailty− (n=11), HIV−/frailty+ (n=10), HIV+/ frailty+ (n=11).

All of the men in that study had positive CD4 and CD8 T cell responses to at least some of the CMV peptides tested. The frequency of TNF-producing T cells was higher in HIV+ than HIV− individuals. The proportion of CMV responsive T cells producing IL-6 was higher in frail compared to non-frail men. Stratification by HIV infection or frailty status showed significant correlations that were absent when the overall study population was evaluated. The HIV+ non-frail group (n=11) showed strong and significant positive correlations between the total number of CD4 T cells producing either IFNγ or TNF with

the number of activated CD4 T cells. The frequencies of CMV-responsive T cells that produced TNF, IL-6, IL-2, and IFNγ were associated with the presence or onset of frailty. The HIV− frail group showed significant correlations between total IFNγ and TNF responses. The HIV+ frail group also showed strong and significant correlations between the frequency of CD4 T cells producing TNF and the number of activated CD4 T cells, and also between the proportion of CD8 cells producing TNF and serum levels of CRP. The frequency of IL-2-responsive CD4 cells significantly predicted the onset of frailty in HIV− non-frail men but not in HIV+ non-frail men (8). These findings suggested that T cell responses to CMV may not only affect the chronic inflammation present in HIV-infected men but may also be a predictor of frailty in HIV− men.

While all of the men in that study responded to CMV peptides, CMV presence in those studied was not investigated. This left unanswered an important question: do differences in the amount of CMV in the circulation account for the differences in the number of CMV-responsive T cells in the circulation? The very low expression of CMV proteins while the virus is latent could prime the immune system, leading to an increase in the number of CMV-responding T cells in the circulation.

That study raised important questions, which this project aimed to investigate. Specifically, this project sought to determine whether the presence of CMV in the blood affects the inflammatory response by T cells to CMV peptides. By detecting and quantifying any CMV DNA present and correlating this with known (i.e., already measured) T cell responses to

CMV peptides and antigens, this project sought to improve the understanding of the role CMV plays the pathogenesis of chronic inflammation.

F. **Aims and Hypotheses**

Based on the previous studies discussed above, this project focused on understanding whether there is a relationship between the detection of CMV in PBMC and the T cell responses to CMV. The first step was to detect CMV in PBMC. Previous studies had detected CMV in PBMC using nested PCR (118) which is a sensitive method but is not quantitative. The development of droplet digital PCR (ddPCR), which was purported to be a highly sensitive and also quantitative technique, led to its use in this project. Recently, ddPCR had been used to detect CMV in blood and semen (112, 119) but at the start of this project, a sensitive ddPCR assay needed to be developed and then applied to the appropriate human biological specimens. Therefore, the specific aims and hypotheses of the project were as follows:

Aim 1

To validate the amount of CMV DNA in PBMC using a novel ddPCR assay. A highly sensitive assay was required to enable the detection of CMV DNA in cells where CMV was not actively replicating. The inability of other PCR methods to reliably detect and quantify CMV indicated that CMV DNA was only present in a limited number of cells during the latent phase of CMV infection. While there are other sensitive methods to detect CMV

DNA, they are not quantitative. Once validated, the assay was used to detect and quantify CMV DNA in a variety of human specimens.

Hypothesis 1

CMV DNA can be detected and quantified in PBMC. The improved sensitivity of the ddPCR assay may detect CMV DNA better than other assays such as nested PCR.

Aim 2

To evaluate the relationship between the detection of CMV DNA and the frequency of CMV-responsive T cells. The detection of CMV DNA in PBMC was compared with T cell responses to CMV using available data from a previous study. The aim includes determining whether HIV and frailty status affects the correlation of CMV DNA detected with the pro-inflammatory cytokine responses to CMV peptide stimulation.

Hypothesis 2

a) The quantity of CMV DNA, and specific CMV genes detected, is correlated with the frequency of CMV-responsive T cells; and
b) This correlation varies by the HIV and frailty status of the host.

II. Methods

A. Study Population and Samples

1. MACS-SHARE Cohort

The SHARE is the Baltimore-Washington DC site of the MACS. MACS was established in 1984 (116, 117) and is the nation's largest and longest ongoing prospective study of the natural and treated history of HIV infection in MSM. Conducted at four sites across the country (Baltimore, Chicago, Los Angeles, and Pittsburgh), MACS has collected biological and behavioral data, and stored biological specimens, from more than 7000 men. MACS participants are about half HIV+ and half HIV−. SHARE has followed about 1800 men. Participants in MACS and SHARE are evaluated semi-annually by laboratory analysis of collected blood samples, physical examinations, and analysis of questionnaires that include medical history, health services, and behavior. Plasma, serum, and viable peripheral blood mononuclear cells (PBMC) are frozen and stored in liquid nitrogen at each semi-annual study visit. HIV serostatus is recorded at each SHARE clinic visit. Seropositivity is defined by a positive ELISA with confirmation by western blot. Since 2007, the frailty status of each participant has been assessed at each semi-annual study visit, using the Fried frailty phenotype (FFP) (44); this phenotype is based on the presence of at least 3 of 5 criteria: weakness as measured by grip strength, low physical activity, slow motor performance measured by walking speed, exhaustion, and unintentional weight loss (39). The criteria are assessed either by answers to a questionnaire or by measuring responses (i.e., walking speed is measured by the amount of time taken to walk 4m, and grip strength is measured with a

dynamometer). In the early years of the study (1984-86), semen samples were also collected and stored.

In this project, frozen PBMC collected as part of the SHARE study were obtained from the Johns Hopkins Bio-Repository. These PBMC were used to validate the droplet digital PCR (ddPCR) assay, to detect and quantify CMV DNA, and to investigate the relationship between the detection of CMV DNA and inflammatory responses in individual donors. In some experiments, freshly-isolated PBMC were used, and in others cryopreserved PBMC were used.

2. Samples to Validate ddPCR Assay

To detect and quantify CMV DNA, a ddPCR assay was used. A series of experiments was conducted to determine the sensitivity and specificity of the ddPCR assay in detecting CMV DNA. To determine the sensitivity of the ddPCR assay, a series of 10-fold serial dilutions of CMV AD169 genomic DNA (Advanced Biotechnologies, Eldersburg, MD) was used. Starting with a concentration of $1x10^4$ copies/μl, the CMV DNA was diluted down to 0.1 copies/μl.

To ascertain the effectiveness of the ddPCR assay in detecting CMV DNA in cells, samples from donors who were known to be either CMV+ or CMV-seronegative (CMV−) were tested. Human Foreskin Fibroblasts (HFFs) (obtained from Dr. Ravit Boger, JHU) were either uninfected or were infected with CMV (Towne strain from Dr. Boger) at a multiplicity of infection (MOI) of 1, 0.1, or 0.01, and DNA was harvested 72-hours post infection (h.p.i). 0.05% Trypsin-EDTA (Thermofisher Scientific, Waltham, MA) was used to detach the

HFFs from the well. The infected HFF were spun down at 300g x 5 minutes before being washed in 1 ml 1x Phosphate Buffered Saline (PBS) (Thermofisher Scientific). After spinning the cells down again, the DNA was isolated from the cells as described later in this section.

To corroborate the ability of the ddPCR to detect CMV DNA, DNA from PBMC whose donors were of known CMV serostatus (obtained from Dr. Sean Leng, JHU) were tested by both ddPCR and by nested PCR (as discussed in section III.C). PBMC from 2 CMV+ and 1 CMV− donors were tested by both methods.

It has been suggested that CMV persists in monocytes (120). To compare levels of CMV in monocytes and PBMC, fresh PBMC were used to isolate monocytes from 4 HIV+ participants. PBMC from a SHARE clinic (which is held in the evenings) were held overnight at 4˚C in RPMI 1640 media (Thermofisher Scientific) with 10% fetal bovine serum (FBS) (Thermofisher Scientific) added. The following morning the adhered monocytes were isolated as described later in this section.

3. Cells Used to Detect and Quantify CMV DNA

To quantify the levels of CMV DNA in CMV+ and CMV− donors, PBMC were obtained from 8 CMV+ and 8 CMV− participants from the Women's Health and Aging Studies (WHAS) II (121) (PI Dr. Sean Leng, JHU), which studies the immune response to influenza immunization. These participants had been tested for CMV antibody and had multiple aliquots of PBMC cryopreserved in liquid nitrogen. The average ages of the CMV+ and CMV− participants were 85 and 85.9 years, respectively. In addition, by nested PCR, CMV

DNA had been detected in the CMV+ participants but not in the CMV− participants (unpublished data).

To maximize the likelihood of finding PBMC that contained detectable CMV DNA and thus could serve as a positive control for the ddPCR assay, PBMC from HIV+ SHARE participants obtained before the advent of highly-active antiretroviral therapy (HAART) were tested by ddPCR. The rationale for testing these participants was that because they had uncontrolled HIV viremia and low CD4 T cell counts, they would be likely to have more CMV DNA in their PBMC than virologically-suppressed participants.

For the same reason, semen obtained from 20 HIV+ SHARE participants early in the study, before the availability of HAART, was studied. Previous studies have shown that CMV is found in high concentrations in the semen, as it can be sexually (119) transmitted. The semen samples were thawed at room temperature before the DNA was isolated along with the DNA from PBMC, as described later in this section.

Also studied were PBMC from 36 SHARE participants who had recently been studied by Margolick *et al.* (8) to investigate the relationship between the T cell responses to CMV antigens and the systemic inflammatory response, as described in section I.E of the Introduction. These participants had already been characterized by their HIV status (19 HIV+ and 17 HIV−) and their frailty status (18 frail and 18 non-frail).

B. Laboratory Methods and Materials

1. Thawing Cells

Frozen PBMC were thawed in a 37˚C water bath. When only a small piece of ice remained in the cryovial, 13 ml warm RPMI 1640 media supplemented with 50% FBS was immediately added in a dropwise fashion as follows: 1 ml, 2 ml, 4 ml, and 6 ml, waiting 1 minute between each round of media. Cells were then centrifuged at 300g for 10 minutes and washed using RPMI 1640 with 10% FBS. Cells were counted using a hemocytometer, using trypan blue exclusion to determine the number of viable cells. The number of viable cells recovered from thawed PBMC that had been stored in the SHARE repository was at least 80% of the total number of cells frozen.

2. Monocyte Isolation from PBMC

As described above (section II.B), PBMC from HIV+ SHARE participants were used to isolate monocytes. From each participant, $20x10^6$ PBMC in RPMI 1640 media supplemented with 10% FBS and 1% Penicillin/Streptomycin (Thermofisher) were divided into (a) $5x10^6$ cells, used to isolate DNA from total PBMC, and (b) $15x10^6$ cells, used to isolate monocytes. For the latter, 1% autologous serum was added to the media, and the cells incubated in 3 wells of a 6-well plate (Thermofisher) overnight at 37˚C. The following morning, the cells were examined under a microscope, and adherent monocytes were identified by their morphologic appearance. The nonadherent cells, which were detached in the media, were aspirated along with the cell culture media, and the adherent cells were washed with 1% PBS (Thermofisher). The adherent cells were removed from the well using a cell scraper, and the DNA was isolated as described below.

3. DNA Isolation

DNA was isolated from cells and semen using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. No more than $10x10^6$ cells or 200 µl semen were used from any given sample. DNA was eluted from the QIAamp mini spin columns in 100 µl H_2O, and the DNA concentration and purity were measured using a nanodrop.

C. CMV Detection and Quantification

1. Overview of PCR

Polymerase chain reaction (PCR) is the amplification of a specific target sequence of DNA through repetitive cycles of denaturing, elongation, and annealing. Using forward and reverse primers that are complementary to the target sequence and a DNA polymerase that elongates DNA by adding nucleotides to the 3' end (e.g., Taq polymerase), the target sequence is amplified many times. Commonly-used PCR methods such as real-time PCR (RT-PCR) and nested PCR have limited sensitivity and are not quantitative. Rather, they rely on the generation of a standard curve to quantify the amount of target sequence detected. Nested PCR is a more sensitive PCR assay than RT-PCR because it uses two separate rounds of PCR to detect the target sequence, where the second round detects sequences found only within the PCR product of the first round. This makes nested PCR more sensitive than RT-PCR, but it is still not a quantitative assay.

ddPCR differs from the other PCR techniques by mixing the sample with oil to make 20,000 nanoliter-sized droplets in such a way that the majority of droplets contains only one molecule of DNA. PCR amplification of the template DNA will occur in each droplet, and lack of competing DNA molecules provides a lower background to the assay, thus making ddPCR highly sensitive. Most DNA droplets containing the target DNA will fluoresce due to primers or probes binding to the specific target DNA sequence. Forward and reverse primers use the double-stranded DNA (dsDNA) binding dye EvaGreen to fluorescently label the target DNA (Figure 2.1). Fluorescently-labeled probes rely on Taq man hydrolysis to cleave the fluorescent reporter (e.g. 6-Carboxyfluorescein (FAM) or hexachlorofluorescein (HEX)) at the 5' end, away from the quencher at the 3' end of the probe (Figure 2.2). The reporter then becomes fluorescent, and therefore detectable, because it is no longer in close proximity to the quencher. Because the potential fluorochromes has been incorporated into the nucleotides, the magnitude of the fluorescent signal is proportional to the amount of amplified product in the sample. The number of fluorescent droplets (i.e., droplets containing the target DNA) is read by the droplet reader, and the number of DNA copies/μl is quantified, as described below. The ability of the droplet reader to count the number of positive and negative fluorescent droplets in a known volume allows the quantification of DNA (copies/μl) without the need for a standard curve.

2. ddPCR: Specific Methods

i. Controls

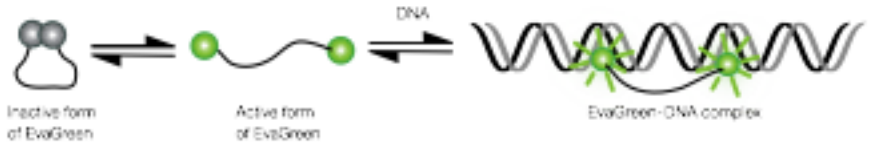
CMV AD169 genomic DNA was used as a positive control and to determine the sensitivity of the ddPCR assay. Negative controls were a no template control (NTC), DNA isolated

from CMV-uninfected HFFs, THP-1 cells, mouse tail DNA, or PBMC from a CMV− leukopak. The CMV− serostatus of the leukopak had been previously determined by CMV ELISA in the Pathology Dept. at Johns Hopkins Hospital. These negative controls were used to determine the background fluorescence levels of the ddPCR assay. The RPP30 gene, of which there are two copies in each cell, was used as a housekeeping control and to allow determination of the exact number of cells contained in each sample tested.

ii. Primers

PCR was set up containing DNA, the probe or primer, and the appropriate supermix for either probes or primers. Forward and reverse primers to IE1 (from collaborator Dr. Leng), UL54 (IDT, Skokie, IL) and UL55 (IDT, Skokie, IL), were designed to bind to specific sequences in their respective CMV genes (sequences in Table 2.1). The same was done for RPP30 primers. The reaction mix was made up of 10 μl of 2X EvaGreen supermix (Bio-Rad, Hercules, CA) that contained the EvaGreen dye. A total of 50 ng DNA and 1 μl of 5 μm forward and reverse primers were used per sample. 3 μl water brought the total reaction mix up to 20 μl per sample (Table 2.1)

Figure 2.1. Mechanism of Fluorescence by the Primers (from (122)).
The primers use EvaGreen, an intercalating double-stranded DNA (dsDNA) binding dye, that fluoresces only when the target sequence is detected (122)



iii. Probe

The FAM-labeled gB probe was obtained from collaborator Dr. Sara Gianella (UCSD). The reaction mix was made up of 10 µl of supermix for probes (Bio-Rad, Hercules, CA) that contained the Taq polymerase, along with 1 µl of gB FAM probe. 1 µl of RPP30 HEX probe (Bio-Rad, Hercules, CA) was included in the reaction mix as a housekeeping control. 5 µl of DNA was included in the reaction mix (Table 2.2).

Figure 2.2. Mechanism of Fluorescence by the Probe (from (122)).
The labeled probe binds to the target sequence amplified by the primer set. During the extension cycles, the 5' fluorescent reporter is cleaved, allowing it to fluoresce as it is no longer adjacent to, and therefore inhibited by, the 3' quencher (122).

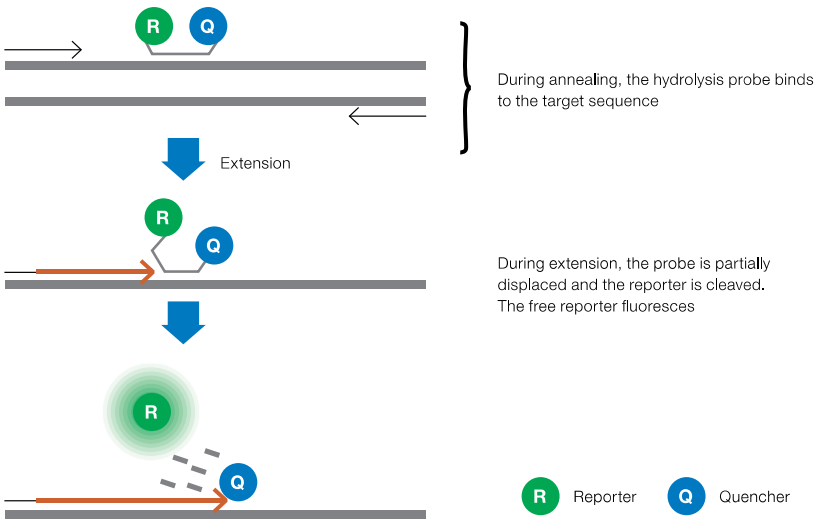


Table 2.1. Primer and Probe Sequences

Primers	IE1 forward primer	TCTGCCAGGACATCTTTCTC
	IE1 reverse primer	GTGACCAAGGCCACGACGTT
	UL54 forward primer	ACGATTCACGGAGCACCAG
	UL54 reverse primer	GCTGACGCGTTTGGTCATC
	UL55 (gB) forward primer	GCGGTGGTTGCCCAACAGGA
	UL55 (gB) reverse primer	ACGACCCGTGGTCATCTTTA
	RPP30 forward primer	GATTTGGACCTGCGAGCG
	RPP30 reverse primer	GCGGCTGTCTCCACAAGT
gB probe set	gB probe set forward primer	AGGTCTTCAAGGAACTCAGCAAGA
	gB probe set reverse primer	CGGCAATCGGTTTGTTGTAAA
	gB FAM probe	AACCCGTCAGCCATTCTCTCGGC with 5'FAM reporter and internal ZEN and 3' IBFQ quenchers
	RPP30 HEX probe	Bio-Rad #10031255

Primers from IDT, Skokie, IL and CMV FAM probe from Dr. S Gianella, UCSD

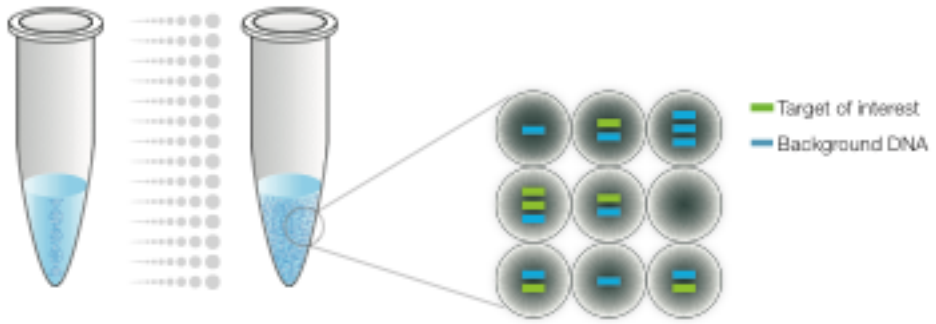
Table 2.2. Primers vs. Probes ddPCR Assay Preparation

Probes	Primers
5 µl DNA (no more than 500 ng total DNA)	5 µl total DNA (10 ng/µl)
10 µl supermix for probes (no dUTP)	10 µl 2X EvaGreen supermix
1 µl CMV FAM probe	1 µl Forward primer (5um)
1 µl RPP30 HEX probe	1 µl Reverse primer (5um)
3 µl H2O	3 µl H2O

iv. Droplet Generation

The ddPCR assay used water-oil emulsion to partition the DNA in the sample into 20,000 nanoliter-sized droplets (Figure 2.3) using the Bio-Rad QX200 Droplet Generator (Bio-Rad, Hercules, CA). 20 µl of sample was added to the middle well of a droplet generator cartridge, and 70 µl of either droplet generator oil for EvaGreen or droplet generator oil for probes was added to the bottom well of the cartridge. When the droplet generation was completed, 40 µl of droplets containing the template DNA were transferred to a 96-well plate and read on the droplet reader, as described below.

Figure 2.3. Droplet generation in ddPCR (from (122))
Single PCR sample partitioned into 20,000 nanoliter-sized droplets. Based on the Poisson distribution, droplets are likely to only get 1 copy of the target sequence which will be detected above the background. Any background fluoresce will be subtracted from the fluorescence of the target sequence (122).



v. PCR Amplification

PCR amplification was performed within each droplet on a thermal cycler. ddPCR cycling conditions for the primers are shown in Table 2.3 and were as follows: initial enzyme activation at 95°C for 5 minutes; 40 cycles consisting of denaturation at 95°C for 30 seconds

followed by annealing and extension at 60°C for 1 minute; signal stabilization at 4°C for 5 minutes; and a final extension at 90°C for 5 minutes.

Table 2.3. Primer PCR Thermal Cycler Conditions

	Temp (°C)	Time	Ramp rate	Number cycles
Enzymes reaction:	95	5 min	2C/sec	1
Denaturation	95	30 sec		40
Annealing/Extension	60	1 min		40
Signal stabilization	4	5 min		1
	90	5 min		1

ddPCR CMV cycling conditions for the probe are shown in Table 2.4 and were as follows: initial enzyme activation at 95°C for 10 minutes; 50 cycles consisting of denaturation at 94°C for 30 seconds followed by annealing and extension at 54°C for 1 minute; and a final extension at 98°C for 10 minutes.

Table 2.4. Probe PCR Thermal Cycler Conditions

	Temp (°C)	Time	Ramp rate	Number cycles
Enzymes reaction:	95	10 min	2c/sec	1
Denaturation	94	30 sec		50
Annealing/Extension	54	1 min		
Signal stabilization	98	10 min		1
Hold	4	∞		

vi. Droplet Reading

After completion of the PCR, the droplets were read on the Bio-Rad QX200 Droplet Reader (Bio-Rad, Hercules, CA), which measures the fluorescence of each droplet and counts the numbers of fluorescent and non-fluorescent droplets. These data were analyzed using QuantaSoft software (Bio-Rad). A threshold that determined which droplets are positive (blue dots in figures) and which are negative (black dots) was manually set in each experiment, based on the level of fluorescence and the proportion of positive droplets obtained in the negative controls, to minimize the background fluorescence levels. This threshold is shown as a pink horizontal line in the figures in the Results section. CMV concentration was quantified by the QuantaSoft software using the following formula:

$$\text{Concentration} = -\ln\left(\frac{N_{neg}}{N}\right)/V_{droplet}$$

where N is the total number of droplets, N_{neg} is the number of negative droplets, and $V_{droplet}$ is the volume of the droplet, calculated by the droplet reader as the droplets are read.

vii. ddPCR Analysis

Background (non-specific) fluorescence levels was subtracted from detected CMV-specific fluorescence. Results were expressed as amount of DNA per million cells based on the cell number determined from copies of RPP30 detected in each sample

3. Nested PCR

125 ng DNA was used for the first nested PCR reaction followed by 5 μl of PCR product for the second round of nested PCR. The IE1 primers used for the second round of nested

PCR were the same as those used for the detection of IE1 by ddPCR (Table 2.5). The NTC and no primer controls (NPC) were used as negative controls. The thermal cycler conditions that were used for the first round and second round of nested PCR are shown in Table 2.6 and Table 2.7, respectively.

Table 2.5. Nested PCR Primers: Round 1 and Round 2

Round 1 primer (forward)	5'-CAATACACTT CATCTCCTCGAAAGG-3'
Round 1 primer (reverse)	5'-ATG GAGTCCTCTGCCAAGAGAAAGATGGAC-3'
Round 2 primer (forward)*	5'-TCTGCCAGGA CATCTTTCTC-3'
Round 2 primer (reverse)*	5'- GTGACCAAGGC CACGACGTT-3'

* Round 2 primers are the same as the IE1 primers used in ddPCR

Table 2.6. Thermal Cycler Conditions Used for Nested PCR Round 1

	Temperature (°C)	Time	# of cycles
Initial denaturation	95	3 min	1
Denaturation	95	30 s	40
Annealing	41.8	30 s	
Extension	72	1 min	
Final extension	72	10 min	10
	12	5 min	5

Table 2.7. Thermal Cycler Conditions Used for Nested PCR Round 2

	Temperature (°C)	Time	# of cycles
Initial denaturation	95	3 min	1
Denaturation	95	30 s	40
Annealing	55	30 s	
Extension	72	1 min	
Final extension	72	10 min	10
	12	5 min	5

4. CMV DNA Data Analysis

To account for differences in the number of cells included in each well, the amount of CMV DNA was divided by the number of cells in the well. To perform analysis of those with and those without detectable CMV DNA, the top 25% of specimens with detectable CMV levels were considered positive and compared to the lower 75% of specimens, which were considered not to contain CMV DNA. Statistical significance of differences between those specimens with detectable CMV DNA and those without detectable CMV DNA was determined using a Mann-Whitney U test, with a p-value less than 0.05 considered significant. Those specimens which were positive for CMV DNA and those without detectable CMV DNA were compared for each CMV gene analyzed (IE1, UL54, UL55, and gB). This data was correlated with data from a previous study quantifying the frequency of T cells producing cytokines in response to CMV peptide stimulation (8). The differences between the groups were measured for each cytokine produced (IFNγ, TNF, and IL-2) in response to each CMV peptide pool used for stimulation. Initially analyzed as a whole group, the group was then stratified by HIV status (2 groups), frailty status (2 groups), and

then by both HIV and frailty subgroups (4 groups): HIV− frailty−, HIV+ frailty−, HIV− frailty+, HIV+ frailty+. The Kruskal-Wallis test was performed to determine if the frequency of T cell responses to CMV peptides when CMV DNA was detected, showed statistically significant differences between the HIV and frailty subgroups.

Spearman correlation coefficients were used to assess the correlation between results obtained using the CMV primers for UL55 DNA and the gB probe, since they measure the same CMV gene, to measure how accurately they measured presence of this gene in a donor. CMV levels measured for a donor using the UL55 primer were plotted against the CMV levels for the same donor using the gB probe. A line of best fit was made between the points and the R^2 was reported.

III. Results

A. An Overview of the Approach

To determine whether CMV plays a role in inflammation, it was necessary to detect and if possible quantify the levels of CMV DNA and RNA, and evaluate how those levels correlate with markers for inflammation. Measuring CMV RNA and protein levels would be an ideal way to measure these correlations. However, in most of the population CMV is in a latent form that is not actively replicating, and the number of cells infected with CMV is very low. Due to these factors, I started by looking for CMV DNA before trying to detect RNA. The ability to detect and quantify CMV DNA would establish which cells are infected with the virus and would permit analysis of possible links to explain the inflammation seen.

Droplet digital PCR (ddPCR) was chosen as a method for analyzing CMV DNA, because it quantifies the amount of DNA per cell and had also been previously used to detect CMV in PBMC (112, 119). By separating the genomic DNA into 20,000 nanoliter-sized droplets that each undergo amplification, ddPCR is very sensitive and good at detecting rare events. Using fluorescent dyes that intercalate with double stranded DNA (dsDNA) (e.g., EvaGreen) or fluorescently tagged probes (e.g., FAM or HEX) that bind to the target gene during the PCR, droplets containing the target DNA of interest fluoresce. Droplets above the background level of fluorescence were considered positive, and using the Poisson distribution the relative numbers of droplets positive and negative for fluorescence allow calculation of the amount of CMV DNA detected in the specimen.

The no-template control (NTC) contained both the forward and reverse primers and the master mix containing the polymerase but no DNA, and thus acted as negative control to measure background (non-specific) levels of fluorescence. The background levels from the negative controls were subtracted from the results of the analyses containing DNA from CMV+ donors, to determine the amount of CMV DNA detected in a given specimen.

To quantify the number of cells in each 20 µl well, RPP30, a housekeeping gene present at 2 copies/cell in all specimens, was measured. The amount of CMV DNA in each well was divided by the number of cells, to obtain the number of CMV DNA copies/10^6 cells.

B. Designing a ddPCR Assay to Measure CMV DNA

1. Validation of the ddPCR Assay

As CMV DNA was expected to be at very low levels and in few cells, the first step was to establish the sensitivity and specificity of the ddPCR assay.

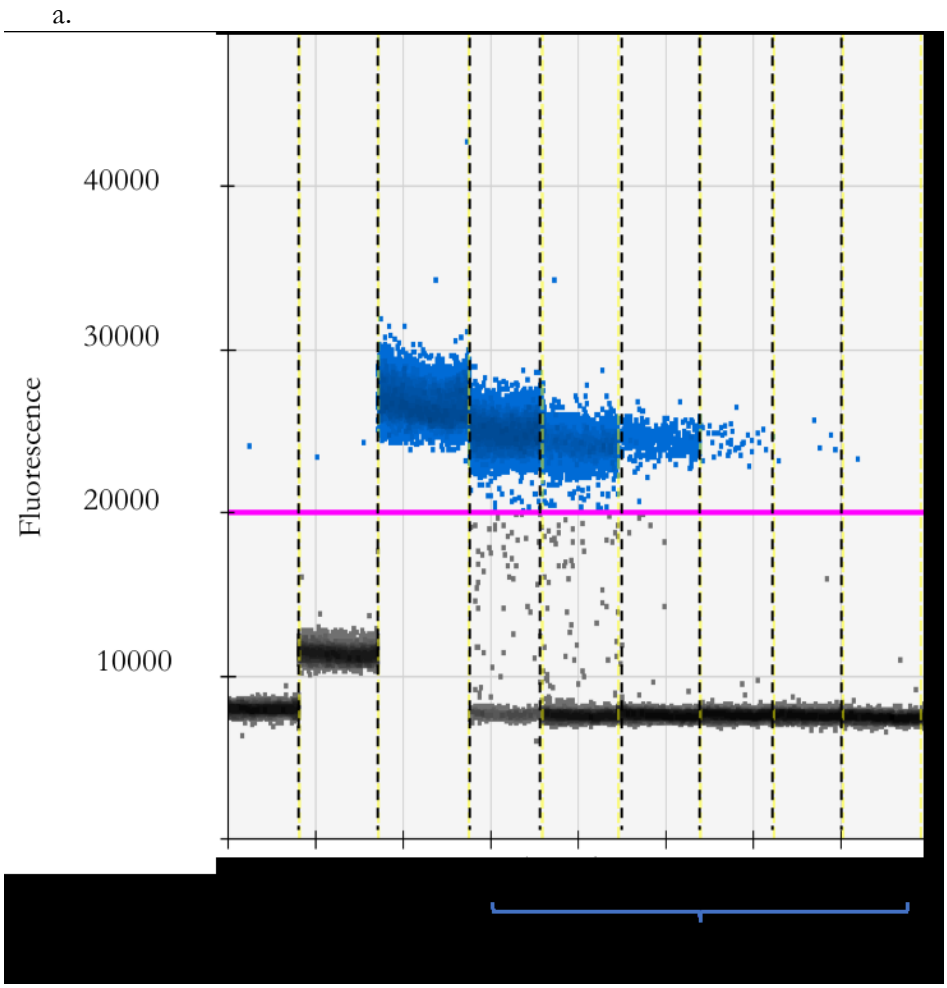
i. Sensitivity of the ddPCR Assay

To determine the sensitivity of the ddPCR assay to detect CMV, a standard curve was created using serial 10-fold dilutions of genomic CMV DNA from the AD169 strain of CMV. The highest concentration of AD169 DNA was $1x10^4$ copies/µl and the lowest was 0.1 copies/µl. 5 µl of the standard was loaded in each 20 µl well. NTC and Human Foreskin Fibroblasts (HFFs) not infected with CMV were used as negative controls, and CMV-infected HFF as a positive control.

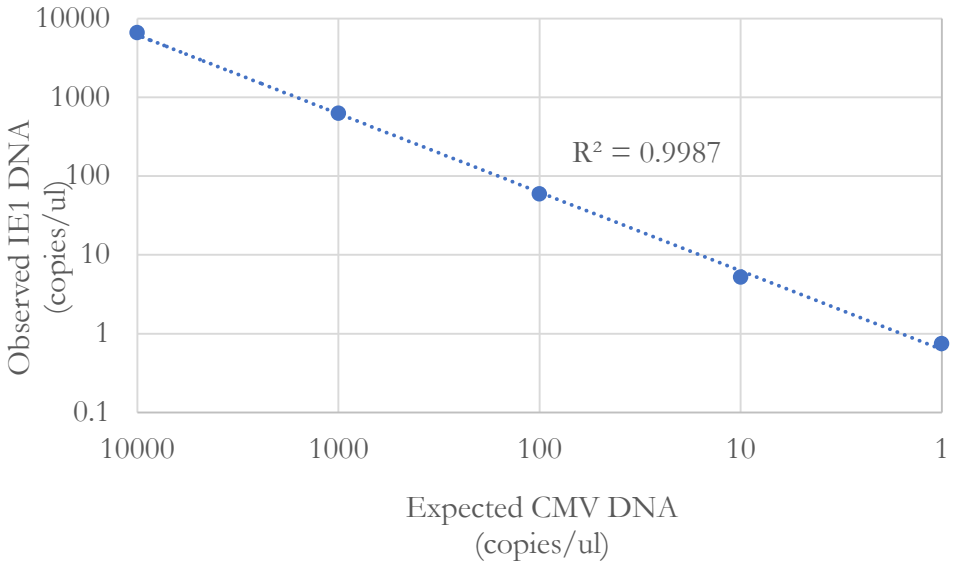
The ddPCR results for this standard curve are shown in Figure 3.1a. The NTC and CMV− HFF controls had 1 and 2 positive droplets, respectively. The average of these values was considered the background level and was subtracted from the other samples. All the droplets in the CMV-infected HFF well were positive. This was because too much template DNA was present, so all the droplets were positive and the sample was oversaturated. Without negative droplets present in the sample, the number of DNA copies/μl cannot be calculated. However, at lower concentrations of CMV DNA this was not a problem, because both positive and negative droplets were present. Further, IE1 was detected from 10^4 CMV copies/μl down to 1 copy of genomic CMV DNA. The dilution with zero copies of CMV had the same number of positive droplets as the negative controls, suggesting that these droplets probably represented non-specific background rather than CMV DNA.

The number of CMV DNA copies measured in each dilution was regressed against the expected number of CMV DNA copies (Figure 3.1b). The R^2 value of this regression was 0.9987, indicating that the assay was highly linear and accurate, with very little variation from the expected values. The sensitivity of the assay was determined as the smallest amount of template that was reliably detected. In this case, the ddPCR assay detected 1 copy of genomic CMV DNA.

Figure 3.1. Standard Curve of Detection of CMV Genomic DNA by ddPCR.
(a) IE1 CMV DNA primers were used to detect CMV DNA in a series of 10-fold serial dilutions of AD169 CMV genomic DNA from 10^4 to 1 copy. No-template control (NTC) and CMV-uninfected Human Foreskin Fibroblasts (HFF) were negative controls. CMV-infected HFFs (HFF+ CMV) were a positive control. 10-fold serial dilution of CMV genomic DNA (starting with 10^4 copies down to 0 copies) along the x-axis. The fluorescence of each droplet is displayed on the y-axis. The pink horizontal line represents the threshold line dividing the positive droplets (blue dots above the threshold) from the negative droplets (black dots below the line); this line was based on the level of fluorescence present in droplets in negative control specimens. The IE1 CMV DNA-positive and -negative droplets show CMV DNA was detected down to 1 copy. (b) Plot of the number of CMV copies measured vs the number of copies expected. The R^2 value of 0.9987 indicates the high accuracy of the assay with little variation from the expected values.



b.

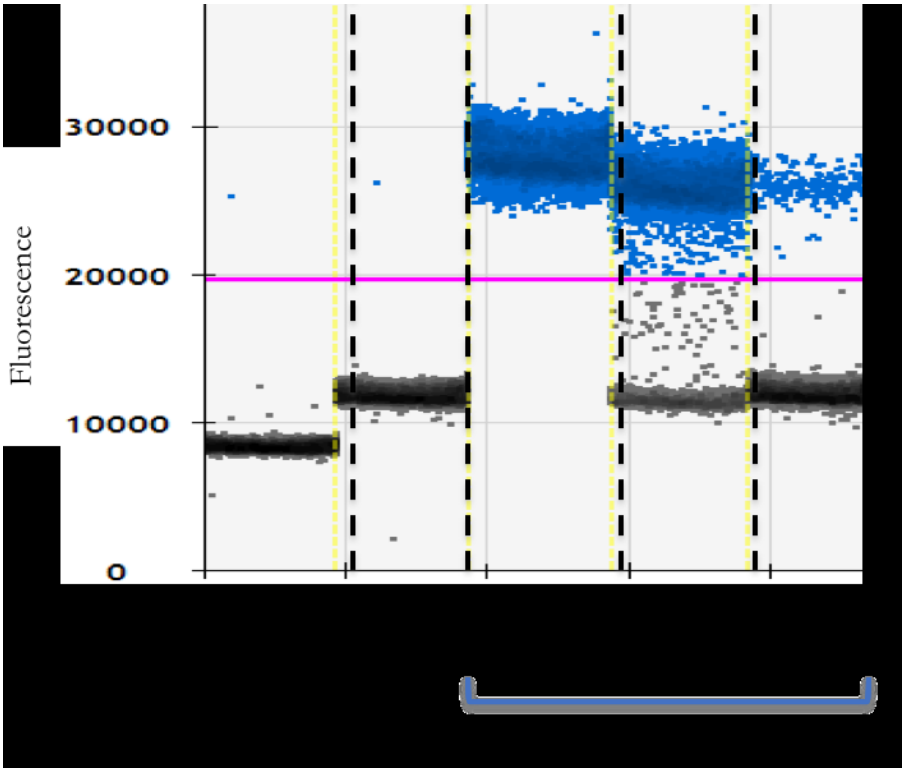


ii. CMV Detection in Infected Cells at Low Multiplicity of Infection (MOI)

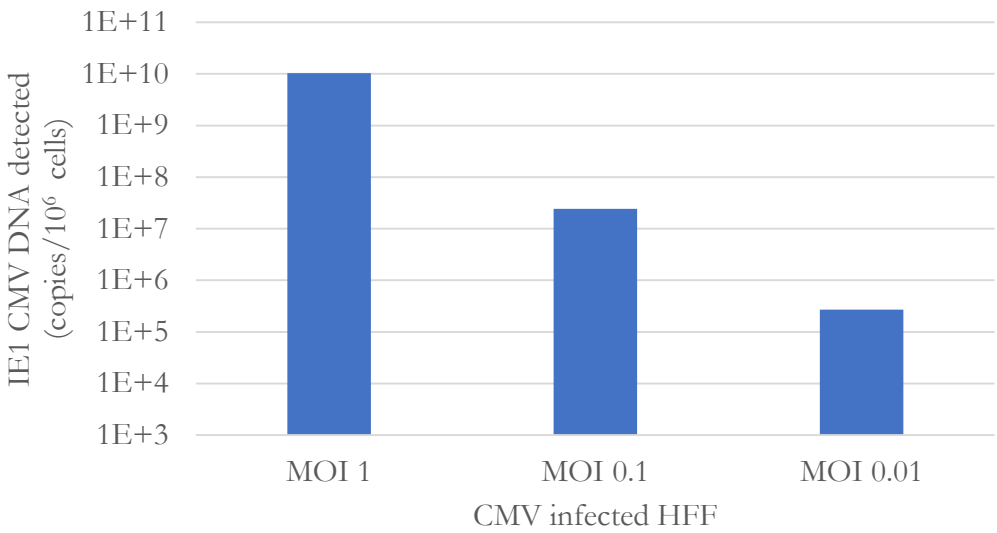
Having shown that ddPCR could detect CMV DNA reliably in a pure DNA preparation, the next step was to determine if ddPCR could do the same in DNA isolated from cells infected with CMV. To test this, HFFs infected at multiple MOIs including low MOIs were used. When MOI=1, 1 viral particle is present for each cell and using the Poisson distribution, a cell has more than a 69% chance of getting 1 or more copies of the virion. Using a MOI=0.01 means infecting cells with 1 virion for 100 cells. This leads to fewer cells being infected, replicating the conditions in PBMC where only few cells are infected with CMV. Using CMV− HFFs and NTC as negative controls, IE1 primers detected CMV DNA at the MOI of 0.01 (Figure 3.2).

Figure 3.2. CMV Detection in HFFs.
(a) CMV detection in HFFs infected with CMV at multiple MOIs. NTC and CMV− HFF were negative controls. 50 ng DNA used for each well from CMV+ HFF at MOI of 1, 0.1 and 0.01. The fluorescence of each droplet is displayed on the y-axis. CMV DNA could be detected at MOIs of 1 to 0.01. (b) The number of CMV IE1 DNA copies/10^6 cells.

a.



b.



iii. Comparison of ddPCR and Nested PCR

To corroborate the accuracy of the ddPCR assay, DNA isolated from human PBMC was probed for IE1 using both the ddPCR and the nested PCR method. Two CMV+ donors (70 and 76) and a CMV− donor (79) were tested. The CMV serostatus of these donors had previously been determined by testing for CMV antibodies by ELISA. Donors 70 and 76 were positive, while donor 79 was negative for CMV antibodies. For the nested PCR, in addition to the NTC, a no primer control (NPC) was also included as another negative control. While the fluorescence threshold for most experiments was set based on the negative controls, in this experiment the threshold was raised to be just beneath the fluorescence level of the positive control. This was done because it was unclear what the cluster of droplets with mid-fluorescence levels were. One possibility was that they could be droplets representing a sequence variant of CMV DNA, where the IE1 primer recognizes the sequence but doesn't bind as effectively as with the primary CMV strain, resulting in a lower level of binding and hence fluorescence. Another possibility was that these droplets could be primer dimers, which occur when the primers bind to themselves; the resulting dsDNA can intercalate with the EvaGreen dye and fluoresce. The lack of this cluster of droplets in all donors suggests that these droplets are probably not primer dimers. However, to ensure that all of the droplets considered positive by the droplet reader were most likely to be CMV DNA, the threshold was raised to exclude the cluster of mid-fluorescence droplets. This represents a conservative measurement of CMV DNA.

IE1 DNA was detected in the two CMV+ donors by both ddPCR (Figure 3.3a) and nested PCR (Figure 3.3c). When the CMV IE1 DNA levels detected by ddPCR were quantified (Figure 3.3b), the amount of CMV IE1 DNA was twice as much in donor 70 compared to

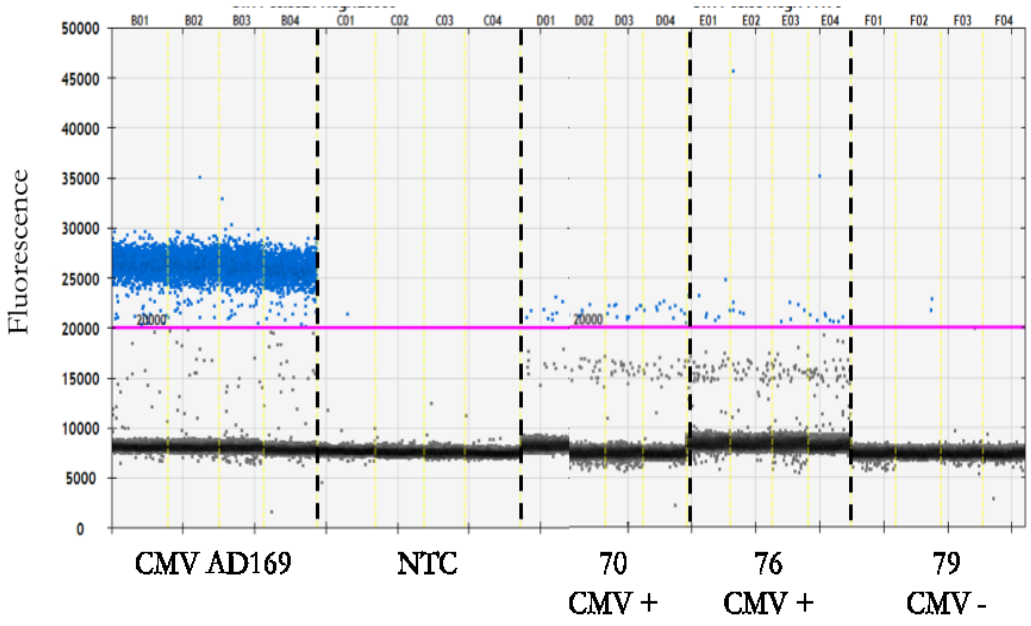
donor 76. The CMV− donor 79 had above-background levels of fluorescence with the CMV IE1 primer, as detected by ddPCR. However, this donor was negative for CMV IE1 DNA by nested PCR and was CMV-seronegative by ELISA (previously performed). Therefore, donor 79 either was really CMV+ with very low levels of CMV DNA that could only be detected by ddPCR, or was a false positive by ddPCR with a high level of background fluorescence.

Figure 3.3. Detection of CMV DNA in PBMC from CMV+ and CMV− Donors. PBMC DNA from CMV+ donors 70 and 76 and CMV− donor 79 were (a) tested and (b) quantified by ddPCR, and (c) tested by nested PCR. (a) CMV AD169 DNA was the positive control and NTC was the negative control. 50 ng DNA from PBMC from CMV+ donors 70 and 76 and CMV− donor 79 was used in each well. Each sample was run in quadruplicate. Fluorescence is displayed on the y-axis. The pink threshold line, demarcating the positive droplets (blue dots above the line) from the negative droplets (black dots below the line), was set based on the positive control to ensure all positive droplets represented CMV DNA.

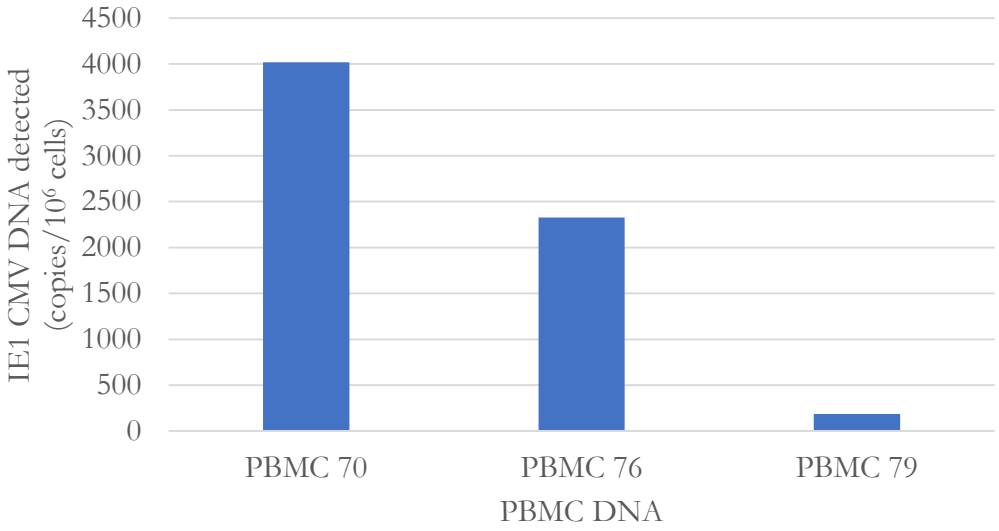
(b) When the amount of IE1 DNA was quantified, after subtracting the background NTC levels, and adjusted for the number of copies of the housekeeping gene RPP30, PBMC from donor 70 (4,017 IE1 copies/10^6 cells) and 76 (2,326 IE1 copies/10^6 cells) were positive for IE1 DNA by ddPCR. PBMC from donor 79 had above-background levels of CMV IE1 DNA (185 IE1 copies/10^6 cells). Although it is possible that this ddPCR has detected CMV DNA at a very low level, the fact that this donor was negative for CMV DNA by nested PCR, and was confirmed to be CMV seronegative by ELISA, suggests that this ddPCR result is a false positive.

(c) to confirm the results of the ddPCR, the PBMC from 2 CMV+ and the CMV− donor were tested by nested PCR. CMV AD169 DNA was the positive control and NTC and no primer control (NPC) was used as negative controls. 125 ng DNA was used for the first nested PCR reaction followed by 5 μl of the PCR product for the second round of nested PCR. The 55 kd band was present in the CMV+ donors 70 and 76 but absent from the CMV− donor 70.

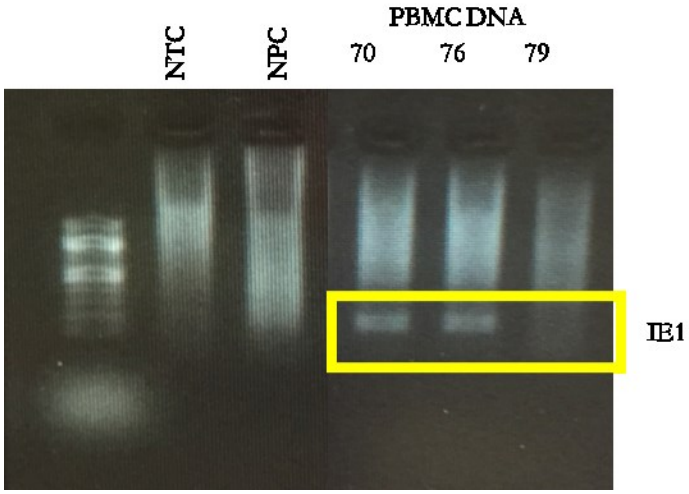
a.


B01 B02 B03 B04 C01 C02 C03 C04 D01 D02 D03 D04 E01 E02 E03 E04 F01 F02 F03 F04
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Fluorescence
20000
20000
CMV AD169
NTC
70
CMV +
76
CMV +
79
CMV -

b.


4500
4000
3500
3000
2500
2000
1500
1000
500
0
IE1 CMV DNA detected
(copies/10^6 cells)
PBMC 70
PBMC 76
PBMC 79
PBMC DNA

c.



iv. CMV Dilutions to Determine DNA Concentration

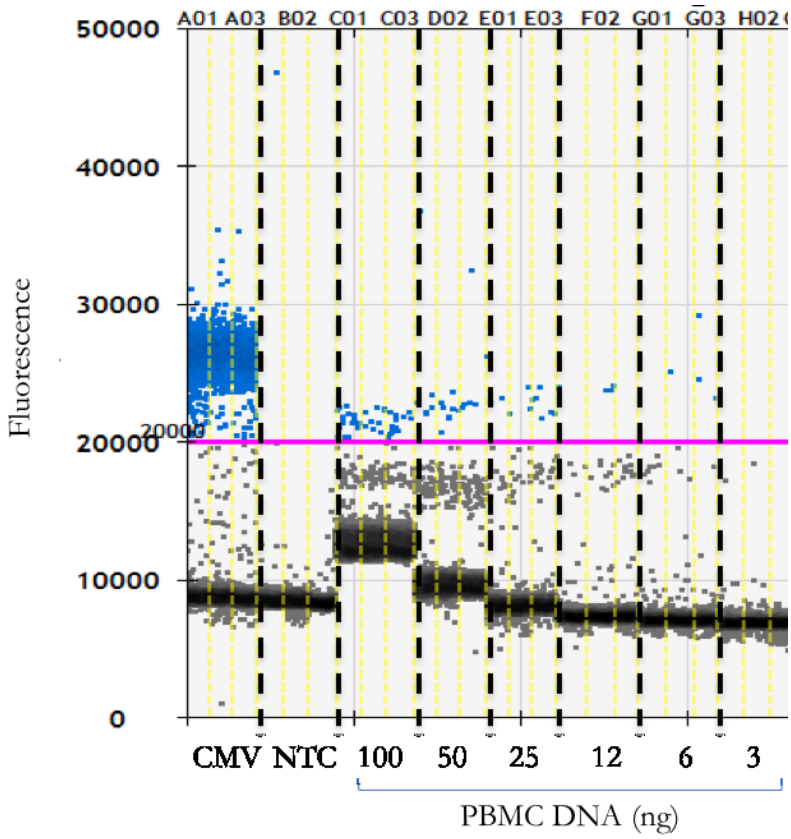
To determine the optimal amount of PBMC DNA to use as the template DNA in the ddPCR assay, serial 2-fold dilutions of PBMC DNA were tested using the IE1 primers. Donor 70 PBMC DNA, that had tested positive for IE1 DNA previously by both ddPCR and nested PCR, was used. Starting with 100 ng DNA, serial 2-fold dilutions down to 3 ng DNA were tested (Figure 3.4a). IE1 CMV could be detected down to 6 ng DNA (Figure 3.4b). The negative droplets in the 100 ng DNA sample had the highest fluorescence compared with the negative droplets in the other samples. High fluorescence of the negative droplets reduced the separation between the positive and negative droplets. Having clear separation of positive and negative droplets is important to help distinguish a true positive droplet from a false positive.

As previously discussed, the clusters of droplets beneath the threshold were not included as positive droplets as they may not be due to CMV DNA. The number of droplets in these

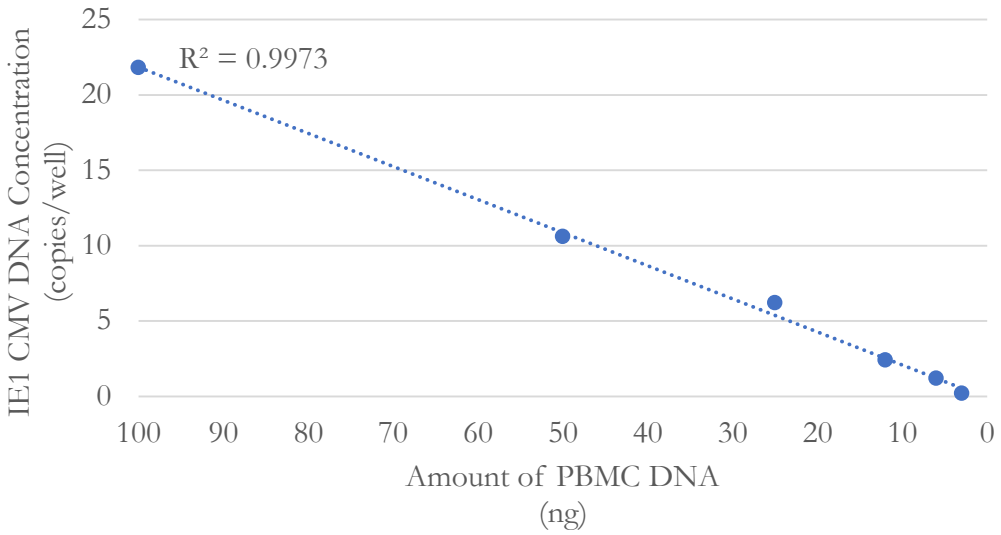
clusters decreased as the concentration of CMV DNA decreased. This implies that the droplet fluorescence in this cluster is related to the amount of DNA detected, rather than a contamination or primer dimers. It is therefore possible that CMV DNA was detected but has a sequence variation. The amount of CMV IE1 DNA copies detected was regressed against the amount of PBMC DNA (Figure 3.4b). The R^2 value of this regression was 0.9973, indicating that the assay is accurately detecting CMV DNA in a highly linear fashion with little variation.

Figure 3.4. Serial Dilutions of CMV DNA to Determine the Optimal Amount of DNA to Use in Each Well for the ddPCR Assay.
(a) 2-fold serial dilutions made from PBMC DNA from CMV+ donor 70, from 100 ng to 3 ng DNA. Samples run in triplicate, with CMV genomic DNA and NTC as positive and negative controls, respectively. (b) Plot of the amount of CMV IE1 DNA measured vs the amount of DNA in each serial dilution. IE1 CMV DNA could be detected from 100 ng to 6 ng DNA. The R^2 value of 0.9973 indicates high accuracy of the assay with little variation from the expected values.

a.



b.



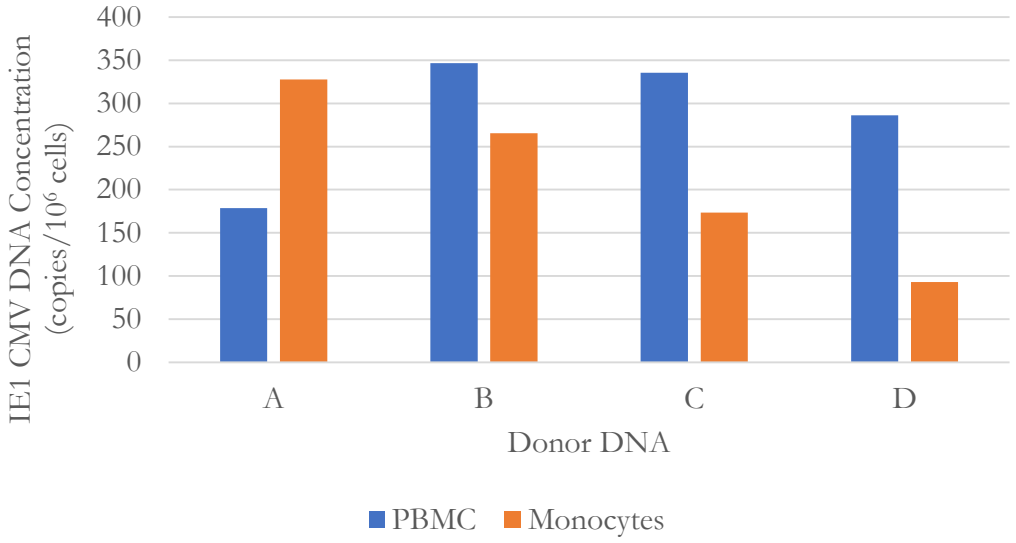
Each of the preceding experiments independently showed that the assay could reliably detect CMV DNA, and these experiments together demonstrated the validation of the ddPCR assay. As discussed in detail above, these experiments showed that the assay was sufficiently sensitive to detect 1 copy of genomic CMV DNA and also that CMV DNA was detectable in CMV+ HFF infected at a low MOI. To corroborate the detection of CMV DNA by ddPCR, another experiment showed that nested PCR could detect CMV DNA in donors where CMV DNA previously had been detected by ddPCR. Together these results lend confidence to the ability of the assay to detect CMV DNA, which was subsequently tested in a variety of specimens.

v. CMV in PBMC Compared to Monocytes

In addition to improving the understanding of the biology of CMV and which cells are persistently infected with CMV, identifying cells with a higher amount of CMV DNA would

help validate the ddPCR assay. It has been suggested that latent CMV virus may reside in monocytes. More CMV DNA may therefore be detected by ddPCR in monocytes than in PBMC. CD14+ monocytes were isolated from PBMC from HIV+ SHARE donors by adhering overnight in RPMI media with autologous human serum to the bottom of a plate. Adherence of the monocytes to the bottom of the well was confirmed by verifying the cell morphology under a microscope. DNA isolated from both PBMC and monocytes from the same donors were tested by ddPCR for CMV IE1 DNA. In 3 out of 4 donors the amount of IE1 CMV DNA detected in monocytes was less than in the PBMC DNA from the same donor (Figure 3.5). These results suggest that CMV DNA was not detected in higher concentration in monocytes compared to PBMC. Therefore, it is unlikely that monocytes are the only cells containing CMV DNA.

Figure 3.5. CMV DNA Detected in PBMC and Monocytes.
CMV IE1 DNA levels detected in PBMC (blue) and isolated monocytes from the same donor (orange) assessed by ddPCR. Monocytes were isolated from PBMC by adhering overnight to the bottom of a well in RPMI supplemented with autologous serum. 50 ng of DNA was used for each donor in duplicate. The NTC was used as previously mentioned to determine the non-specific background level. The levels of IE1 CMV DNA are expressed as copies/10^6 cells. In 3 out of 4 donors, IE1 CMV DNA was greater in PBMC than in isolated monocytes.



2. Detection and Quantification of CMV DNA Levels

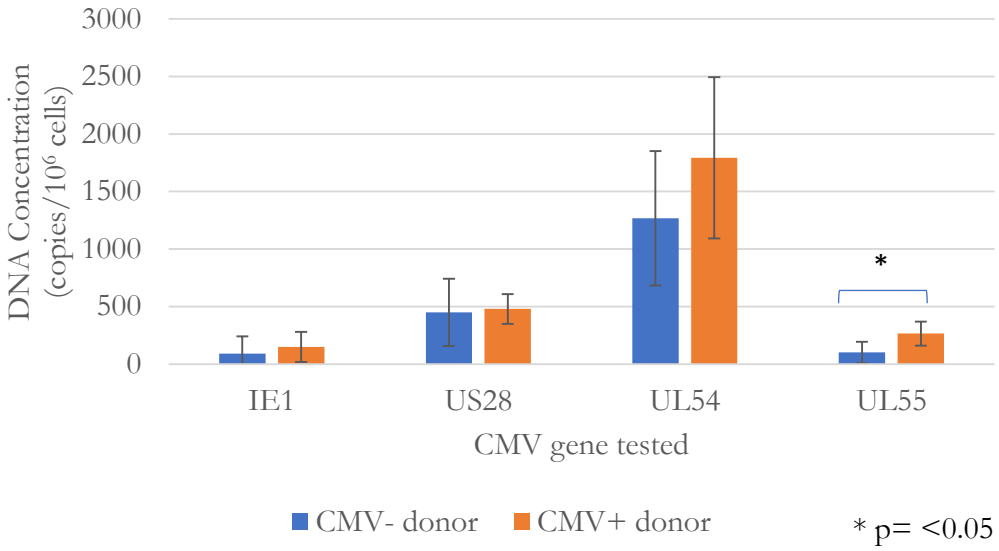
i. The Effect of CMV Serostatus on Detection of CMV DNA

To determine whether the amount of CMV DNA in a sample correlates with CMV serostatus of the donor of that sample, DNA from PBMC from CMV+ and CMV− donors was tested by ddPCR. CMV+ and CMV− donors were identified from a cohort of elderly individuals who had enrolled in a longitudinal study investigating the immune response to influenza vaccination. 8 CMV+ and 8 CMV− donors with multiple PBMC samples stored in liquid nitrogen were studied. The average ages of the CMV+ and CMV− donors were 85 and 85.9 years, respectively. DNA isolated from PBMC was tested with primers for CMV IE1, UL54, UL55, and US28. UL54 and UL55 primers were used because they were reported to be very sensitive and specific for detecting CMV DNA (123). US28 was used because it encodes a latently expressed gene. Although DNA was being tested for, rather than RNA or protein expression, the expected latent nature of CMV in PBMC suggested that targeting a CMV gene expressed during latency, such as US28, may improve the chances of detecting the CMV DNA.

More UL55 DNA was detected in CMV+ donors compared to CMV− donors, and this difference was significant ($p=0.04$ by a Mann-Whitney U test; Figure 3.6a). IE1 and UL54 CMV DNA were also more abundant in the CMV+ donors, but not significantly so ($p>0.05$), although the difference in levels of UL54 DNA was of borderline significance ($p=0.07$) (Figure 3.6b).

Figure 3.6. CMV DNA Detected in CMV+ and CMV− Donors.
CMV DNA levels in CMV+ donors compared to CMV− donors. (a) PBMC DNA from 8 CMV+ donors (blue) were compared with PBMC DNA from 8 CMV− donors (orange). Serostatus of all donors had previously been confirmed in Dr. Leng's geriatric study by an antibody test and by nested PCR. DNA was isolated from PBMC from all donors and IE1, US28, UL54, and UL55 DNA was quantified by ddPCR. Bars indicate the medians, and error bars indicate the interquartile range. Significantly more UL55 CMV DNA was detected in the CMV+ donors than in the CMV− donors (shown by * which denotes p=<0.05). IE1 and UL54 DNA were also higher in the CMV+ donors, but not significantly so. (b) The median for each CMV gene in each group is shown, along with the p-value for the difference between the CMV+ and CMV− groups

a.



b.

	Median CMV− (copies/10^6 cells)	Median CMV+ (copies/10^6 cells)	P-value
IE1	92.69	150.40	0.29
US28	450.56	480.67	0.87
UL54	1267.79	1793.31	0.07
UL55	101.84	265.86	0.04

ii. Background Fluorescence Levels of CMV Primers and Probes in CMV− Donors

To determine if the positive droplets seen in CMV− donors from previous experiments truly represented CMV DNA or were false positives, specimens from known CMV− donors were tested for CMV DNA by ddPCR. These included mouse tail DNA, DNA from CMV− THP-1 cells from different days in culture (day 1 and day 9), and PBMC from a CMV− leukopak. Multiple primers were tested: IE1, UL54, and UL55, and a primer/probe set that detected CMV gB DNA. The gB probe had been used in previous studies and was thought to be more specific than the primers (119). As discussed in the Methods section, the probe and primers use different mechanisms of binding to the target gene and generating fluorescence in the ddPCR droplet.

Specimens from the CMV− donors were tested with the primers in duplicate with 4 NTC samples, and with the gB probe in triplicate with 9 NTC wells. The threshold for positive fluorescent droplets was set above droplets in the NTC or beneath the CMV AD169 genomic DNA in the positive control. Setting the threshold at this level, the NTCs of the IE1 (Figure 3.7a), UL54 (Figure 3.7b), and UL55 (Figure 3.7c) primers had totals of 1, 9, and 4 positive droplets, respectively (out of more than 15,000 in most cases) in the 4 NTC replicate wells. The gB probe (Figure 3.7d) had no positive droplets in any of the 9 replicate wells.

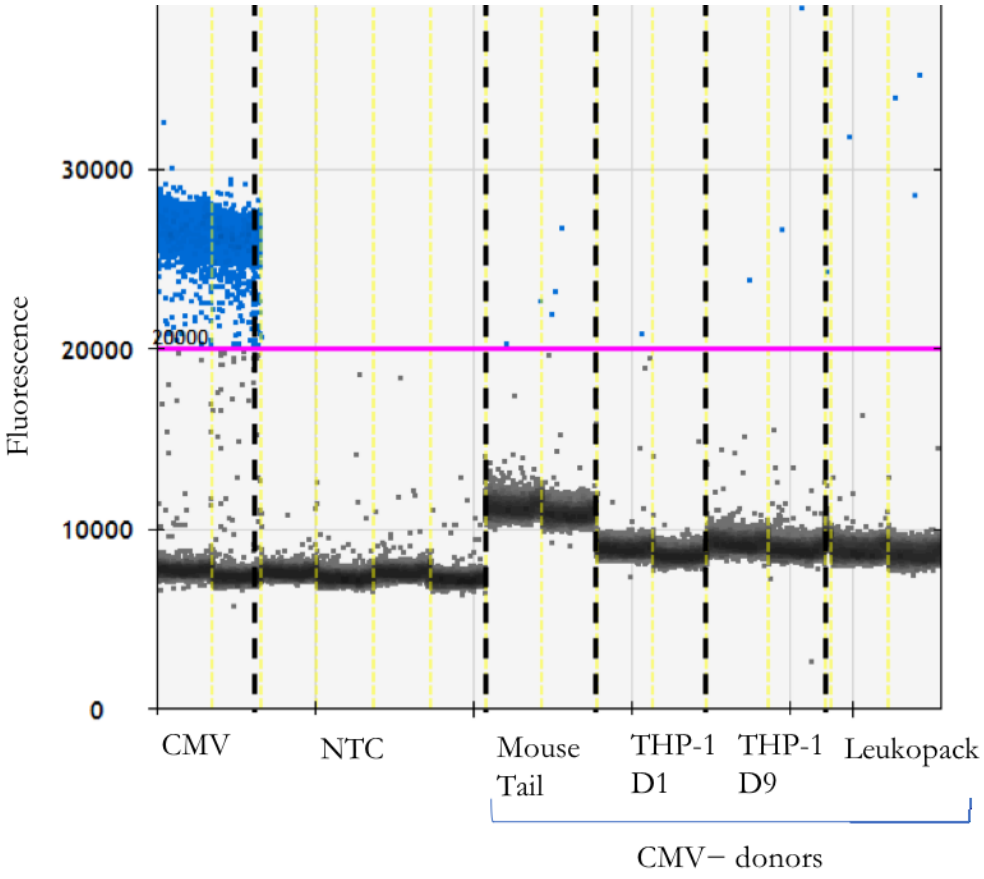
UL54, UL55, or the gB probe showed no positive fluorescent signal in the THP-1 D1 and the leukopak DNA (Figure 3.7e). In contrast, mouse tail DNA and the THP-1 D9 cells were positive for all primers at very low levels. The background (non-specific) level of the

IE1 primers was very low in each CMV− sample tested, with a few positive droplets present. The UL55 primers showed no nonspecific background as they were undetectable in the THP D1 and the leukopak, and only a very low level of nonspecific positivity was present in the THP D9 sample. The THP-1 D9 also showed a small positive fluorescent signal for the gB probe which was absent in all the other samples tested. Results were expressed as the number of copies/10^6 cells for all specimens, except for mouse tail DNA which does not contain the RPP30 gene used to normalize to the number of cells in human specimens.

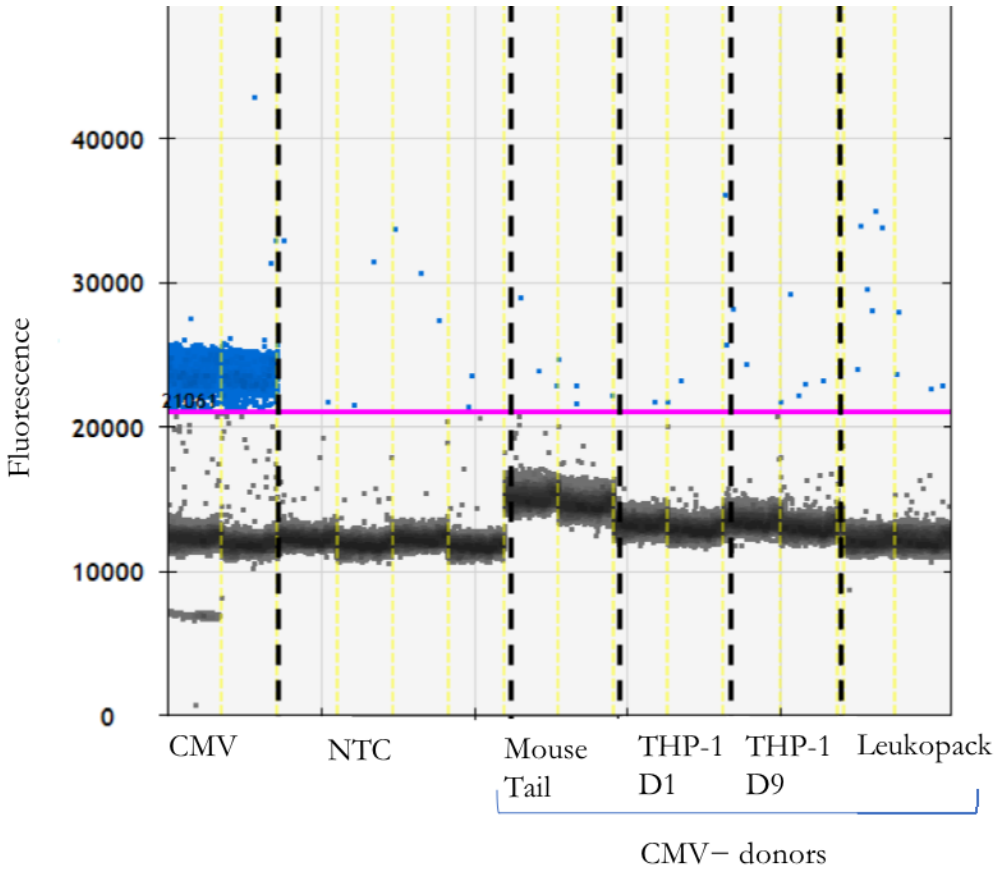
These data demonstrated that, to accurately quantify the amount of rare CMV DNA, it is necessary to take into account false-positive or background levels of fluorescence, especially when using the CMV primers. The gB probe showed better specificity than the primers and no background in the majority of donors tested. Therefore, for subsequent experiments in this project, multiple negative control samples in duplicate or triplicate were used in addition to the NTC, especially when using the primers. An average of the nonspecific background levels measured in the CMV− samples was calculated for each primer. This average background level in known CMV− samples was subtracted from the amount of CMV DNA recorded in the studied samples.

Figure 3.7. Non-Specific Fluorescence of Droplets in CMV− donors.
CMV− donors: Mouse tail, CMV− THP-1 cell DNA from day 1 of culture (THP1 D1) and day 9 of culture (THP1 D9), and CMV− leukopak DNA were tested for CMV DNA using the (a) IE1, (b) UL54, and (c) UL55 primers. CMV AD169 genomic DNA was the positive control and NTC was the negative control. Samples were run in duplicate with 4 NTC wells. The threshold for positivity of droplet fluorescence was set based on the fluorescence of droplets in the NTC. A few positive droplets were present in all CMV− specimens. (d) Testing the specimens from the CMV− donors with the gB probe in triplicate with 9 NTC wells. No positive droplets were present in specimens from CMV−donors, except THP1-D9. (e) Depiction of the quantified levels of positive signal above NTC background. The lack of RPP30 in mouse tail prevented the calculation of the amount of DNA/10^6 cells. The y-axis represents the amount of background positive signal measured/well. Positive fluorescent signal was absent for the UL54 and UL55 primers in DNA from THP1-D1 and the leukopak PBMC. Mouse tail showed positive fluorescent signal for IE1, UL54, and UL55 primers. The IE1 primer showed positive fluorescent signal in all the samples tested. The gB probe showed no positive fluorescent signal in except in the THP-1 D9 cells, demonstrating that the gB probe was more specific than the primers.

a.



b.


40000
30000
20000
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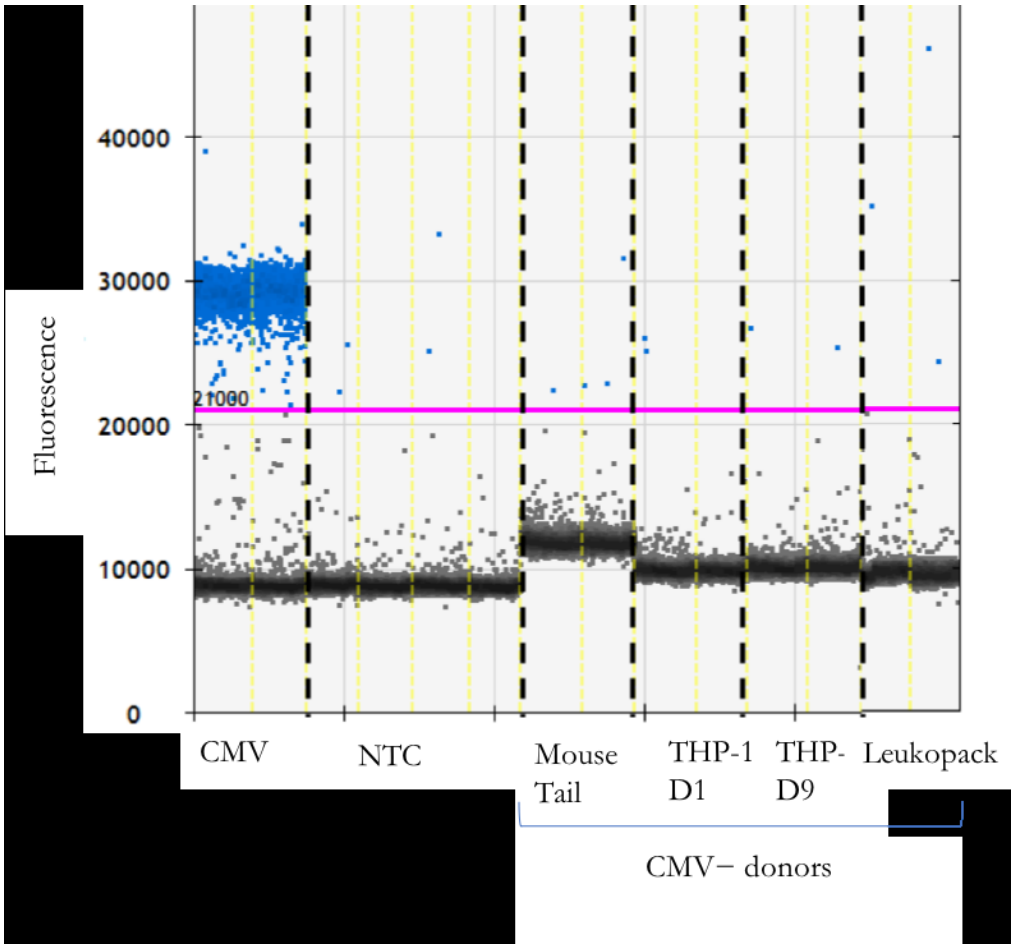
0
Fluorescence
21063
CMV
NTC
Mouse Tail
THP-1 D1
THP-1 D9
Leukopack
CMV− donors

c.


40000
30000
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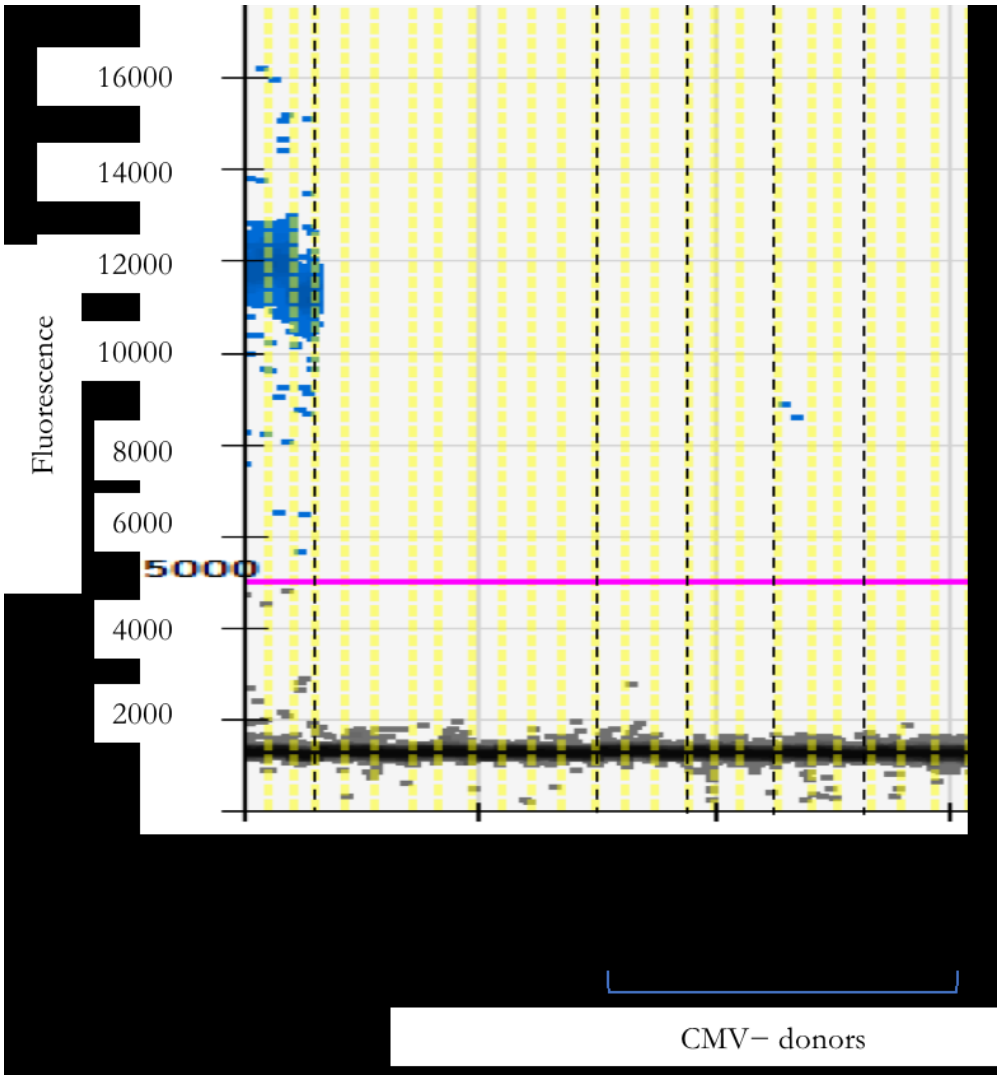
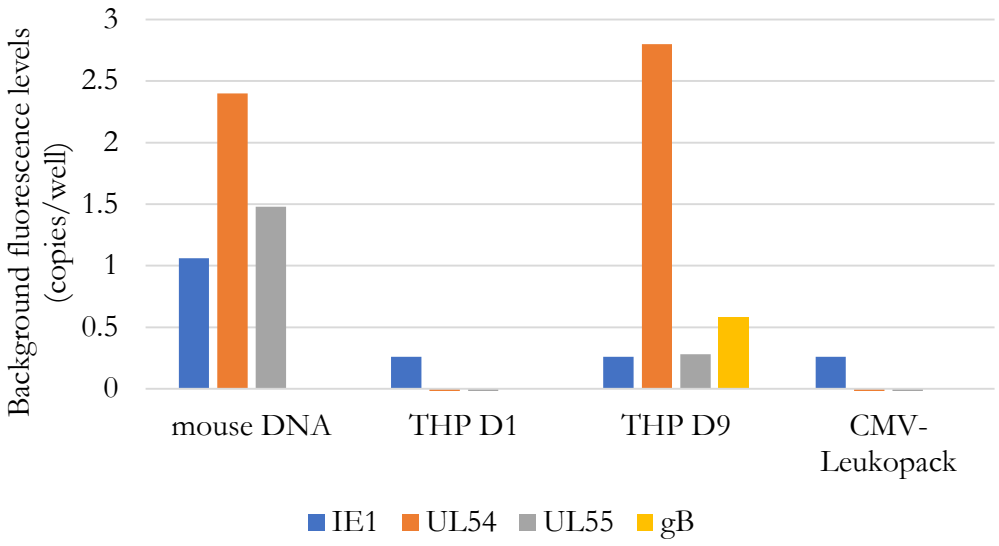
Fluorescence
21000
CMV
NTC
Mouse Tail
THP-1 D1
THP-D9
Leukopack
CMV− donors

d.


16000
14000
12000
10000
8000
6000
5000
4000
2000
Fluorescence
CMV− donors

e.



iii. CMV DNA Detection in Semen From HIV+ Men Not Receiving HAART

With CMV DNA rare and hard to detect in people latently infected with CMV, and with the presence of very low-level fluorescence using the CMV primers in the ddPCR assay, validation of the assay as used above required that a human specimen with unequivocal CMV DNA content be found, to serve as a true positive control. To this end, it was decided to test semen from HIV+ individuals not receiving HAART for CMV DNA. We reasoned that because HAART suppresses HIV viremia to undetectable levels, people with HIV infection not receiving HAART have higher HIV viremia and more impairment of cellular immunity than people receiving HAART. Therefore, people not receiving HAART would be less likely to be able to control CMV replication than people receiving HAART, and might have more CMV DNA in their semen. To confirm that the ddPCR assay could detect CMV in semen, semen from 20 HIV+ men not receiving HAART was studied using the gB probe.

gB DNA was detected in 9 of the 20 semen samples (Figure 3.8a). In the 9 samples where gB DNA was detected, the median was $1.4x10^4$ copies/10^6 cells. The ability to clearly detect CMV DNA in a biological sample and not just in the pure CMV genomic DNA or the CMV-infected HFF, was a big step forward for the project. It showed that the assay worked and CMV DNA could be detected. These semen samples with detectable CMV DNA also served as a valuable positive control for future experiments, alongside the CMV AD169 genomic DNA.

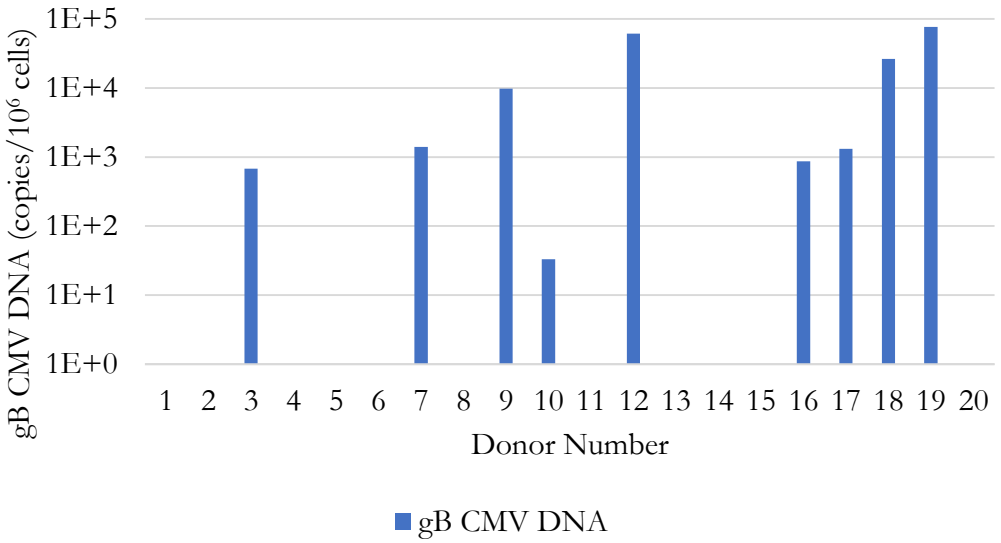
To test whether the primers and the probe were equally sensitive and specific, results obtained using the primers (IE1, UL54, and UL55) were compared to those obtained using the gB probe (Figure 3.8b) in 13 donors. These 13 semen samples selected included 5 with the highest levels of gB DNA detected, and 8 that had undetectable gB CMV DNA. This was to test whether the primers detected CMV DNA in samples that were positive for gB DNA, supporting the data obtained using the probe. It also tested whether the primers could detect CMV DNA in samples that were negative for gB DNA, which would either corroborate the gB probe data or show any differences between the primers and the probe.

CMV DNA was detected in all 13 semen samples. IE1 DNA was detected in 11 samples (median= $0.5x10^4$ copies/10^6 cells), and UL54 (median= $0.8x10^4$ copies/10^6 cells), and UL55 (median= $1.7x10^4$ copies/10^6 cells) were detected in 10 donors. The median amount of gB DNA detected in the 5 donors was $9.8x10^4$ copies/10^6 cells). All semen samples but 2 had multiple target genes detected, and the two remaining samples had only UL55 DNA detected in both cases. The levels of CMV DNA detected by the UL55 primer and the gB

probe were strongly correlated (R^2 = 0.99) (Figure 3.8c). This strong correlation supports the accuracy of the assay to detect CMV DNA, because the UL55 primer and the gB probe target DNA sequences in the same CMV gene. However, the probe detected CMV DNA in only half as many donors as the primer, 5 and 10 out of 13, respectively. The probe is more specific than the primer as shown by the much lower background levels from the probe. However, the primer may be more sensitive than the probe and detect CMV DNA at low levels. It is also possible that lower specificity of the primer means that some of the UL55 DNA detected may be false positive. This may occur at the lower level of detection, but when UL55 and gB are both detected, the levels of CMV DNA measured are very similar.

Figure 3.8. CMV DNA Detection in Semen from HIV-Infected Donors Not Receiving HAART.
(a) CMV DNA detection by gB probe in semen from 20 donors. x-axis is each donor (1-20), the y-axis is CMV DNA (copies/10^6 cells). gB was detected in 9 out of 20 donors. (b) CMV DNA detected by primers: IE1 (blue), UL54 (orange), UL55 (grey), and gB probe (yellow). CMV DNA was detected in all 13 semen samples. (c) Correlation between the amount of CMV DNA detected by the UL55 primer (x- axis) vs the amount detected by the gB probe (copies/10^6 cells) (y-axis). In specimens in which CMV DNA was detected by both the primer and the probe, these values were strongly and linearly correlated (R^2= 0.99).

a.



b.

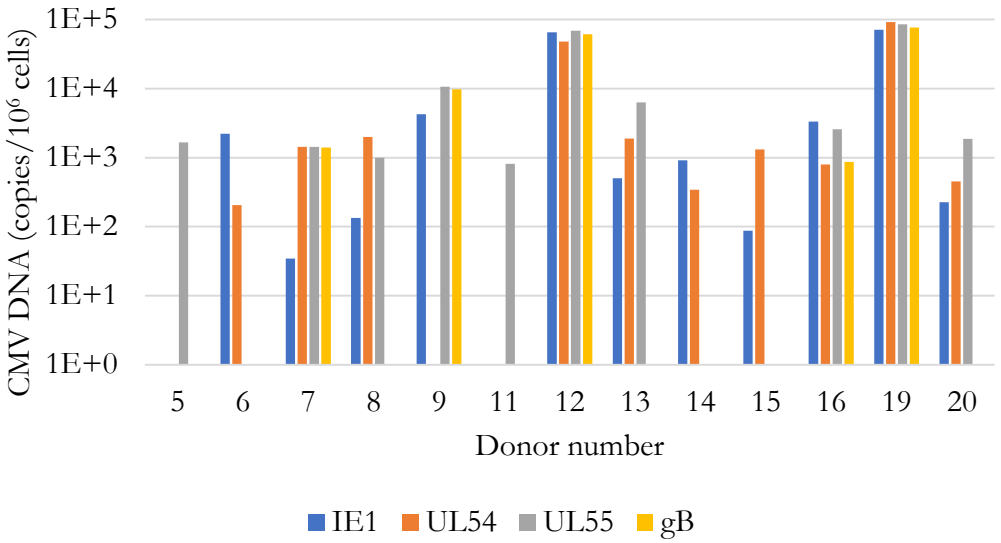
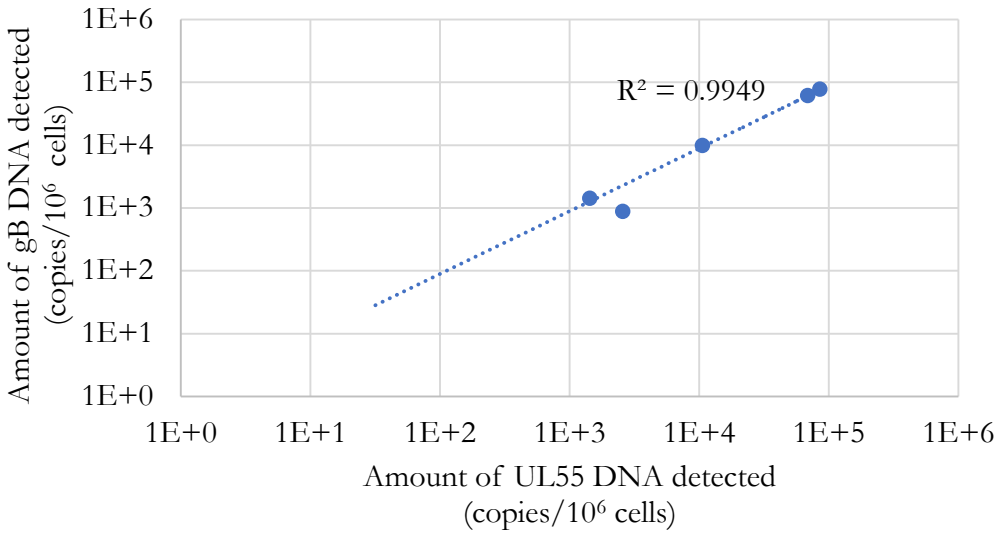


Table 3.1. CMV DNA Detected by CMV Primers and Probe in Each Donor.

	Median amount of CMV DNA detected (copies/10^6 cells)			
Donor	IE1	UL54	UL55	gB
5	0	0	1655	0
6	2218	205	0	0
7	35	1429	1429	1404
8	133	2000	1000	0
9	4251	0	10627	9767
11	0	0	816	0
12	65446	47863	68921	60837
13	501	1894	6295	0
14	917	344	0	0
15	88	1316	0	0
16	3313	798	2577	870
19	70740	91945	85420	76723
20	226	452	1864	0

c.



iv. CMV DNA Detection in PBMC from HIV+ Men Not Receiving HAART

Having detected CMV DNA in semen from a number of HIV+ individuals not receiving HAART, we asked whether PBMC from HIV+ individuals not receiving HAART also had detectable CMV DNA. As previously mentioned, these individuals not receiving HAART had higher HIV viremia and more impaired cellular immunity. This makes them less likely to control CMV replication and therefore more likely to have CMV in their PBMC. Therefore, cryopreserved PBMC from 20 HIV-infected SHARE participants originally obtained when they were not receiving HAART were tested for CMV DNA by ddPCR using the gB probe and the IE1, UL54, and UL55 primers. A semen sample from an HIV-infected donor not receiving HAART, which had previously been shown to be positive for all CMV genes tested by the primers and the probe, was also used as a positive control alongside the CMV AD169 genomic DNA.

Using the gB probe, CMV DNA was detected in PBMC from 16 out of 20 donors (Figure 3.9a). The median amount of gB CMV DNA detected was 44.9 copies/10^6 cells (Figure 3.9b). However, some donors showed much higher levels of CMV DNA in their PBMC, with the median of the 4 highest responders being $2.3x10^4$ copies/10^6 cells. The use of HIV+ donors not receiving HAART enabled the detection of CMV DNA and provided samples that could be used in future experiments as a biological positive control, in addition to AD169 genomic DNA. The findings showed that the assay could detect CMV DNA in PBMC. This enabled the project to continue and investigate the relationship between CMV DNA and the T cell response to CMV.

The 4 donors with the highest levels of gB DNA detected were subsequently tested with the CMV primers IE1, UL54, and UL55 to determine if the probes and primers gave similar results (Figure 3.9c). UL54 and UL55 DNA were detected at similar levels compared to the gB probe, with median values: $3.2x10^4$ copies/10^6 cells and $3.4x10^4$ copies/10^6 cells, respectively, but levels of IE1 DNA were slightly lower at an average of $0.7x10^4$ copies/10^6 cells.

The gB probe identified clusters of droplets with differing fluorescence levels, all above the background levels, but less than the positive control AD169 DNA. These could be sequence variations in different strains than the gB probe can identify. The lack of background from the probe allows the identification of these clusters and the confidence that these droplets represent CMV DNA.

The amounts of CMV DNA detected using the different CMV primers and the gB probe were also very similar. Comparing the amount of CMV detected using the UL55 primers with the quantity of CMV detected using the gB probe (Figure 3.9d) for each donor also showed a very strong correlation ($R^2 = 0.92$). This was expected, because the gB probe and the UL55 primers target the same UL55 gene.

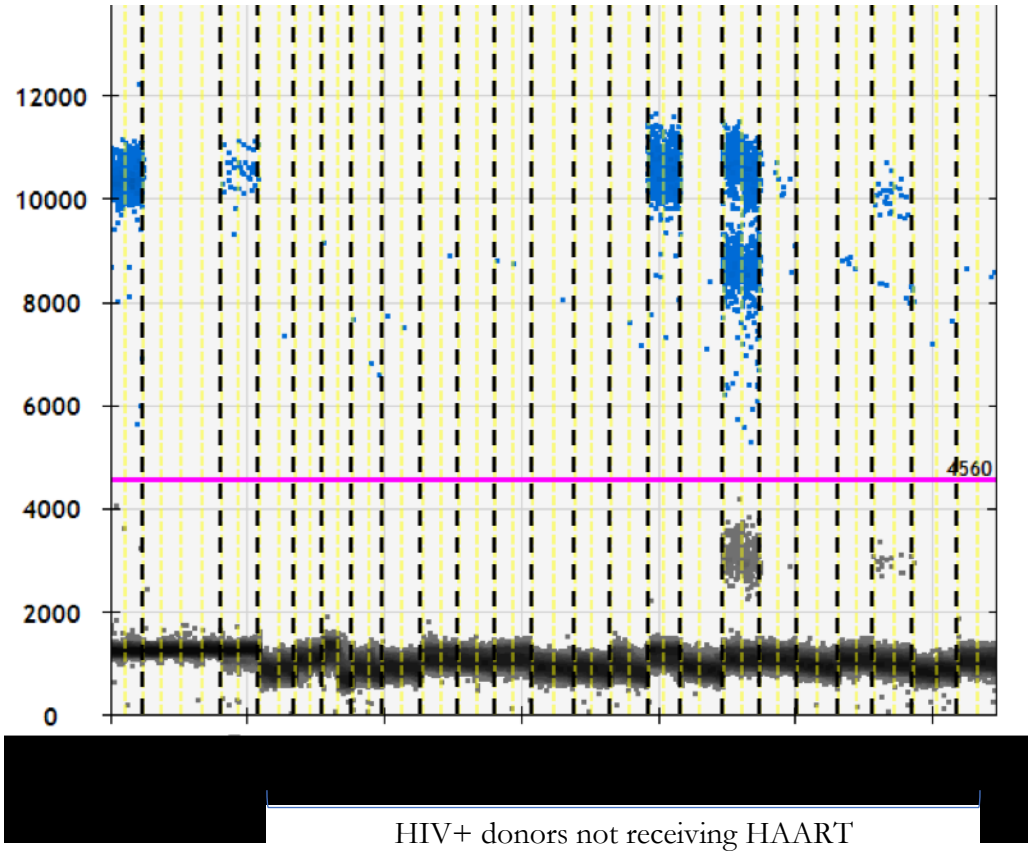
The results of these experiments showed that the ddPCR assay could quantify CMV DNA in PBMC and semen from donors. CMV DNA was detected in greater amounts in CMV+ donors compared to CMV− donors. In non-suppressed HIV-infected donors who were not receiving HAART, CMV DNA could be detected and quantified in both PBMC and semen. As discussed previously, the amounts of UL55 DNA and gB DNA detected in both PBMC and semen showed a strong correlation when both were detected in the same sample. In addition to the donors for whom gB DNA was detected, the UL55 primer detected CMV DNA in some semen samples from donors that did not yield detectable gB DNA. The latter donors had lower amounts of CMV DNA than the donors who had both UL55 and gB DNA (Table 3.1). This may be due to differences in the sensitivity of the UL55 primer compared to the probe. Although the gB probe has a high specificity, shown by the lack of non-specific background fluorescence levels in CMV− samples, it is possibly less sensitive than the UL55 primers and may miss some CMV DNA only detected at a low level. The lower specificity of the UL55 primers, shown by the higher background levels in CMV− samples than the gB probe, raised the possibility that donors with only UL55 DNA detected at a low level may be false positives. However, multiple DNA targets were detected in most donors and similar amounts of CMV DNA were detected by each primer and the probe. Although the background levels were higher with the primers, a positive signal above

background could still be measured in the PBMC and semen from these HIV-infected donors not receiving HAART.

The conclusion of these experiments was that ddPCR could quantify CMV DNA in PBMC. Therefore, the next step in this project was to determine if the detection of CMV DNA in PBMC was correlated with the T cell responses to CMV in the same donors.

Figure 3.9. CMV DNA Detection in PBMC from 20 HIV-Infected Donors Not Receiving HAART.
(a) 50 ng DNA from PBMC from 20 HIV+ donors not receiving HAART was tested by ddPCR using the gB probe for CMV DNA in duplicate. Each HIV+ donor not receiving HAART is along the x-axis. CMV AD169 and 50 ng DNA from semen from a non-suppressed HIV-infected donor were used as the positive controls. 4 NTC wells were the negative controls. The threshold for the gB probe was set above the negligible NTC background level and above the cluster of droplets with low fluorescence, as it was unclear whether they represented true positives for CMV DNA fluorescence. Other clusters of droplets with different fluorescence were considered positive and quantified as detectable CMV DNA. (b) The amount of gB DNA copies/10^6 cells. The average amount of gB DNA detected (7674 copies/10^6 cells) from the 20 donors with error bars showing the standard deviation. (c) Graph showing the CMV levels in PBMC from the 4 donors with the highest amount of gB detected. These PBMC were tested with IE1 (blue), UL54 (orange), and UL55 (grey) primers. The amount of gB (yellow) DNA detected is also shown. The average amount of CMV DNA detected by each primer and the probe is shown, with error bars indicating the standard deviation. Similar amounts of CMV DNA were detected with the primers and with the probe. (d) Graph correlating the CMV DNA levels measured with the UL55 primer and with the gB probe. The R^2 value of 0.9225 shows how linear this correlation was, with little variability.

a.


12000
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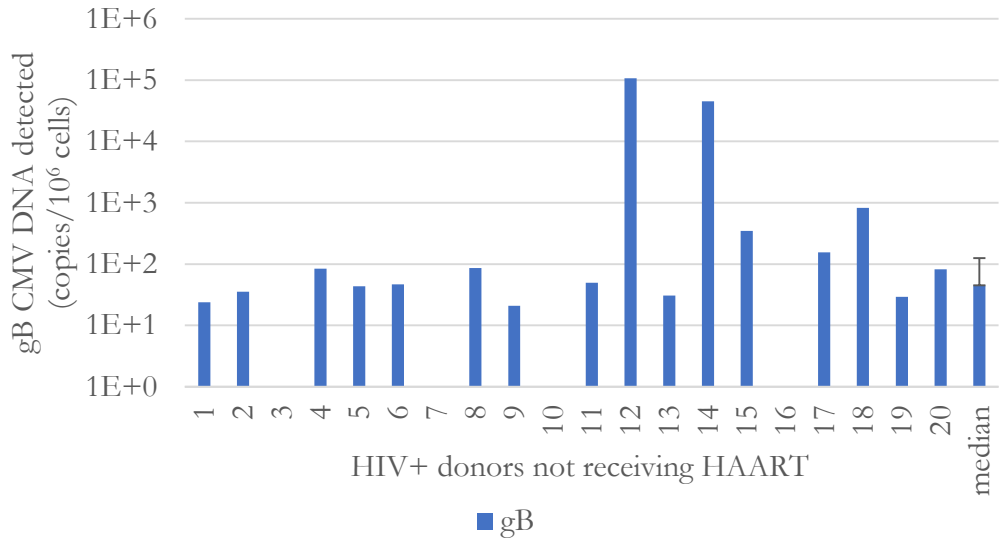
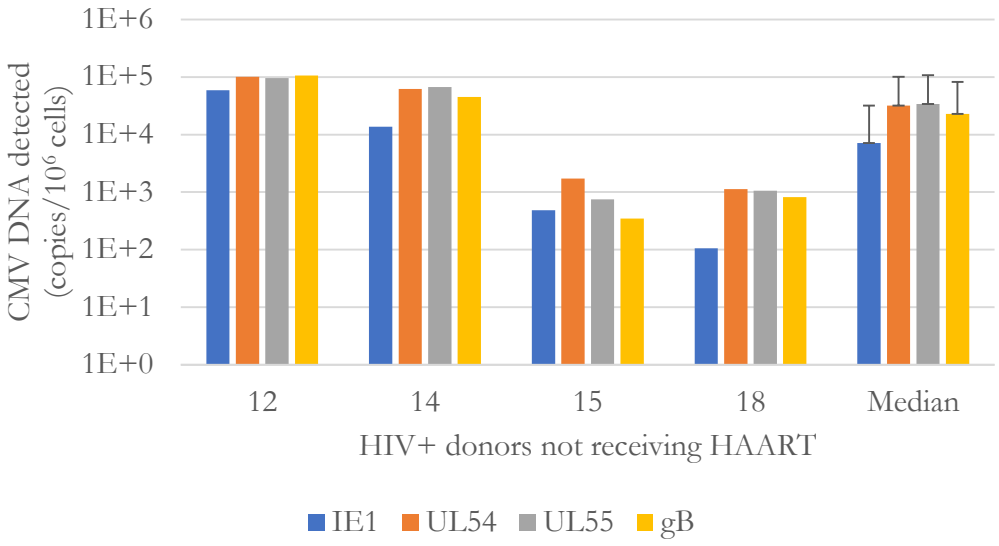
8000
6000
4000
2000
0
4560
HIV+ donors not receiving HAART

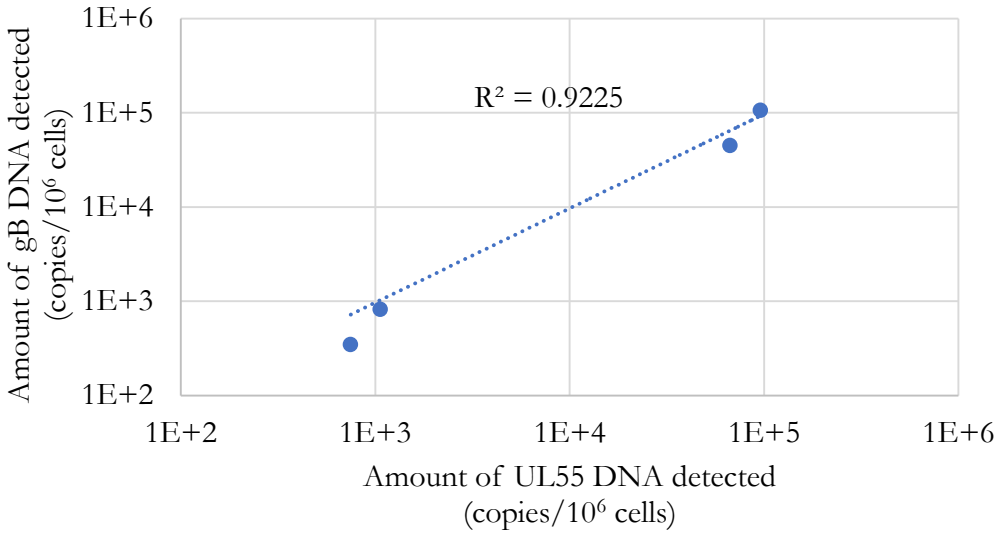
b.


1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
1E+0
gB CMV DNA detected (copies/10^6 cells)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 median
HIV+ donors not receiving HAART
gB

c.


1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
1E+0
CMV DNA detected (copies/10⁶ cells)
12
14
15
18
Median
HIV+ donors not receiving HAART
IE1
UL54
UL55
gB

d.


1E+6
1E+5
1E+4
1E+3
1E+2
Amount of gB DNA detected (copies/10⁶ cells)
R² = 0.9225
1E+2
1E+3
1E+4
1E+5
1E+6
Amount of UL55 DNA detected (copies/10⁶ cells)

C. The Relationship Between CMV DNA Levels and T Cell Responses to CMV

Having established and validated an assay to quantify CMV DNA in PBMC from various types of human donors, including virologically suppressed HIV+ donors receiving HAART, the next aims were to determine: (1) whether the amount and type of CMV DNA in PBMC were associated with the T cell response to CMV; and (2) if so, whether these associations were correlated with HIV status or frailty status.

To address these aims, CMV DNA was measured in PBMC from donors whose T cell responses to CMV had previously been measured (8). In the previous study, PBMC from 42 SHARE participants were stimulated with overlapping peptides that covered 19 CMV ORFs, and the proportion of CD4+ and CD8+ T cells producing IFNγ, TNF, and IL-2 in response to this stimulation was measured. This broad range of peptides was used because of the wide range of CMV antigens that can stimulate CMV-specific T cells (48). These subjects also presented a diversity of HIV status (half +, half −) and frailty status (half +, half −), making them an ideal group for the present study.

The amount of CMV DNA in PBMC from these donors was quantified. The CMV primers (IE1, UL54, and UL55) and the gB probe quantified differences in the amount of each CMV gene detected. The T cell responses to CMV were compared between those donors with detectable CMV DNA and those without CMV DNA. A Mann-Whitney U test was used to determine significance of differences between those donors with and without detectable CMV DNA.

1. Presence of CMV DNA in PBMC from 36 SHARE Participants Analyzed

Of the 42 donors in the previous study, 6 did not have cryopreserved PBMC available for testing. Therefore, 36 donors were tested: 19 were HIV+ and 17 were HIV−; 18 were frailty+ and 18 were frailty−. The 6 donors that were not tested included: 3 HIV+ and 3 HIV− and 3 frailty+ and 3 frailty−. In the group as a whole (Table 3.2), CMV DNA was detected in 30 out of 36 donors (83.3%): DNA for IE1 was detected in 10 donors (27.8%), for UL54 in 28 donors (77.8%), for UL55 in 20 donors (55.6%), and for gB in 15 donors (41.7%).

Table 3.2. Characteristics and Results for 36 Donors Tested for CMV DNA.
HIV− (n=17) and HIV+ (n=19); frailty− (n=18) and frailty+ (n=18); HIV-frailty subgroups: HIV−frailty− (n=9), HIV−frailty+ (n=8), HIV+ frailty− (n=9), HIV+ frailty+ (n=10)

Donor	Age	HIV Serostatus	Frailty Status	Presence of IE1 DNA	Presence of UL54 DNA	Presence of UL55 DNA	Presence of gB DNA
35	64	-	-	-	+	+	+
39	66	-	-	-	+	+	+
41	65	-	-	+	+	+	-
44	49	-	-	-	+	+	+
50	63	-	-	-	+	-	+
52	70	-	-	+	+	+	+
54	62	-	-	-	-	-	-
68	70	-	-	-	-	-	-
69	73	-	-	+	+	+	
32	62	-	+	-	+	+	-
33	54	-	+	+	+	+	-
36	56	-	+	+	+	+	+
40	65	-	+	-	+	-	-
43	64	-	+	+	+	+	+
53	66	-	+	-	+	-	-
61	69	-	+	-	-	-	-
63	67	-	+	-	-	-	+
26	55	+	-	+	+	+	+
30	63	+	-	-	+	-	-
37	54	+	-	-	+	+	-
46	64	+	-	-	+	+	-
48	65	+	-	-	+	+	-
55	51	+	-	+	+	-	-
58	61	+	-	-	+	-	-
66	61	+	-	+	-	+	+
70	61	+	-	-	+	-	-
29	52	+	+	-	+	+	+
31	59	+	+	-	+	-	-
38	69	+	+	-	+	+	+
42	57	+	+	-	-	+	-
47	55	+	+	-	+	+	+
49	58	+	+	-	+	+	+
56	68	+	+	-	+	-	-
60	52	+	+	-	-	-	-
64	66	+	+	+	+	-	+
65	64	+	+	-	-	-	-

HIV seropositive= +, HIV seronegative= −; frailty+ = positive for Fried frailty phenotype, frailty− = negative for Fried frailty Phenotype; IE1/UL54/UL55/gB DNA + = CMV DNA detected, IE1/UL54/UL55/gB DNA − = CMV DNA not detected

2. The Relationship Between Concentrations of CMV DNA, HIV Status, and Frailty Status

When analyzed by HIV status (Figure 3.10a), CMV DNA was detected by the UL54 and UL55 primers and by the gB probe (Table 3.3). However, the differences between the amount of CMV DNA detected in the HIV+ and the HIV− groups were not significant for any of these genes. The average amount of gB DNA detected was 5 times higher in HIV− donors compared to HIV+ donors but this difference was not significant. Similarly, concentrations of UL54 and UL55 DNA were higher in HIV+ compared to HIV− donors, but not significantly so. Median IE1 DNA concentrations were not above background in either HIV− or HIV+ donors.

CMV DNA concentrations did not differ by frailty status (Figure 3.10b) for any of the CMV genes studied (Table 3.4). Levels of DNA detected were generally higher in the non-frail compared to the frail donors. The average amount of UL55 DNA was 3 times higher in frailty− donors compared to frailty+ donors, and with a p-value of 0.13 the difference between the groups was close to being significant. Similarly, UL54 DNA concentration was higher in frailty− donors compared to frailty+ donors but was not a significant difference. The median concentrations of IE1 and gB DNA were not above background in either the frailty− or the frailty+ donors. However, high levels of CMV DNA, especially gB DNA, were detected especially in frailty− donors.

CMV DNA concentrations for each gene tested did not differ between each HIV-frailty subgroup (Figure 3.11). Although the median concentration of IE1 DNA (Figure 3.11a) was

not above background in any subgroup (Table 3.5), IE1 DNA was detected in donors, especially donors in the HIV+ frailty− and the HIV− frailty+ subgroups. The difference in IE1 DNA concentration between the HIV+ frailty− and the HIV+ frailty+ subgroups was almost significant (Table 3.6).

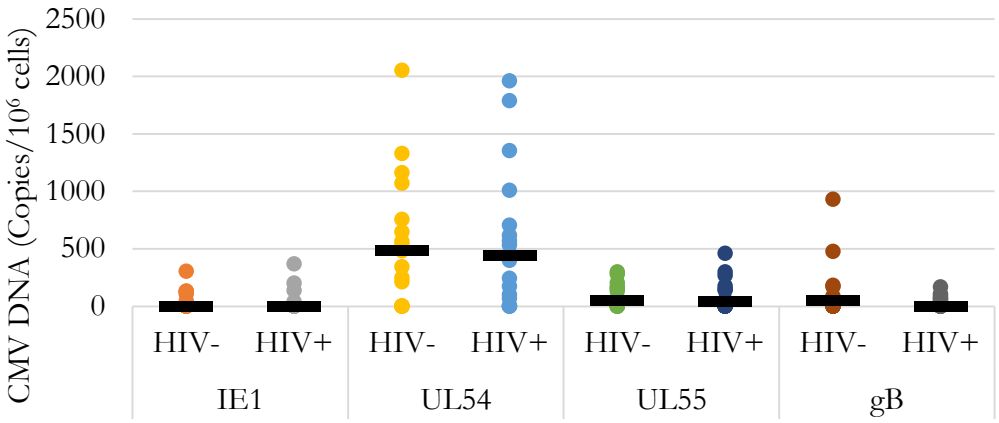
UL54 DNA was detected in each HIV-frailty subgroup (Figure 3.11b), although none of the differences were significant (Table 3.8). Nevertheless, higher concentrations of UL54 DNA were detected in frailty− donors of both HIV+ and HIV− serostatus (Table 3.7).

In addition, the amount of UL55 DNA (Figure 3.11c) and gB DNA (Figure 3.11d) showed no significant differences between any of the subgroups (Tables 10 and 12). For UL55 DNA, the median amount was 3-6 times higher in the HIV− frailty− subgroup compared to the other subgroups (Table 3.9). While the median amount of gB DNA was not detectable in HIV− frailty+ and HIV+ frailty− donors (Table 3.11), the difference between the amount of gB DNA in the HIV− frailty− and the HIV+ frailty− subgroups was almost significant.

Figure 3.10. CMV DNA Levels in PBMC from Donors Stratified by HIV and Frailty Status

Amount of CMV DNA for each primer (IE1, UL54, and UL55) and probe (gB) was compared by (a) HIV serostatus and by (b) frailty status. The black lines represent the median for each group. There were no significant differences between either the HIV+ and HIV− or between the frailty+ and the frailty−, for any CMV gene.

a.



CMV DNA detected in HIV− and HIV+ for each CMV gene

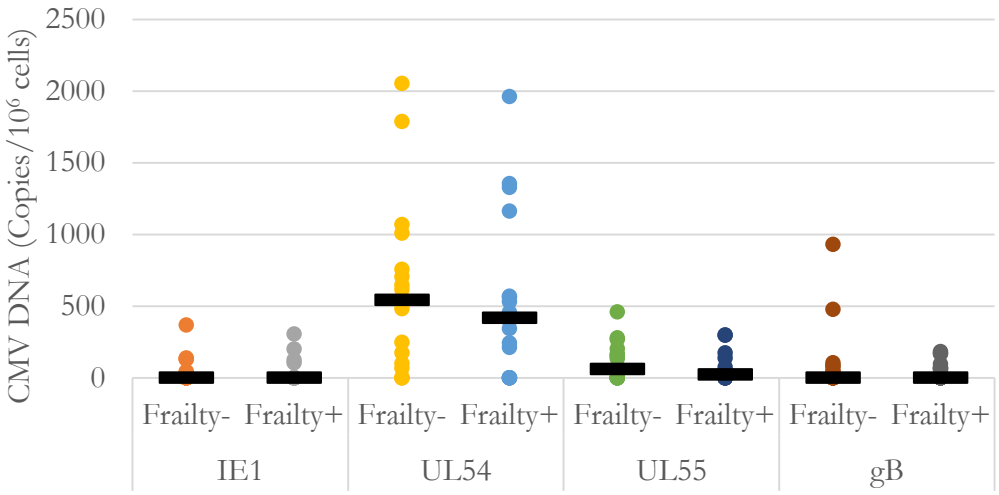
Table 3.3. Median Amount of CMV DNA Detected for Each CMV Gene by HIV Serostatus

The p-value determines if there was a significant difference between the HIV+ (n=19) and the HIV− (n=17) group for each CMV gene.

CMV gene	HIV serostatus	Median CMV DNA detected (copies/10^6 cells)	IQR	P-value
IE1	−	ND	0 – 105.1	0.75
	+	ND	0 – 0	
UL54	−	482.1	0 – 756.0	0.75
	+	439.0	83.9 – 661.0	
UL55	−	46.9	0 – 156.6	0.9
	+	42.6	0 – 143.4	
gB	−	46.2	0 – 108.7	0.3
	+	ND	0 – 61.6	

HIV− = HIV seronegative; HIV+ = HIV seropositive; ND= Not Detected; IQR= interquartile range

b.



CMV DNA detected in frailty− and frailty+ for each CMV gene

Table 3.4. Median Amount of CMV DNA Detected for Each CMV Gene by Frailty Status

The p-value determines if there was a significant difference between the frailty+ (n=18) and the frailty−(n=18) group for each CMV gene.

CMV gene	Frailty status	Median CMV DNA detected (copies/10^6 cells)	IQR	P-value
IE1	−	ND	0 – 43.8	0.5
	+	ND	0 – 0	
UL54	−	541.7	118.9 – 743.3	0.5
	+	418	52.8 – 567.2	
UL55	−	60	0 – 164.2	0.1
	+	21.7	0 – 67.2	
gB	−	ND	0 – 74.5	0.9
	+	ND	0 – 70.2	

Frailty− = negative for Fried frailty Phenotype; frailty+ = positive for Fried frailty Phenotype; ND= Not Detected; IQR= interquartile range

Figure 3.11. CMV DNA Levels in PBMC from Donors Stratified by HIV-Frailty Subgroup for Each CMV Gene Tested

(a) IE1, (b) UL54, (c) UL55, and (d) gB. CMV DNA was detected in each HIV-frailty subgroup with no significant differences between the subgroups.

a.

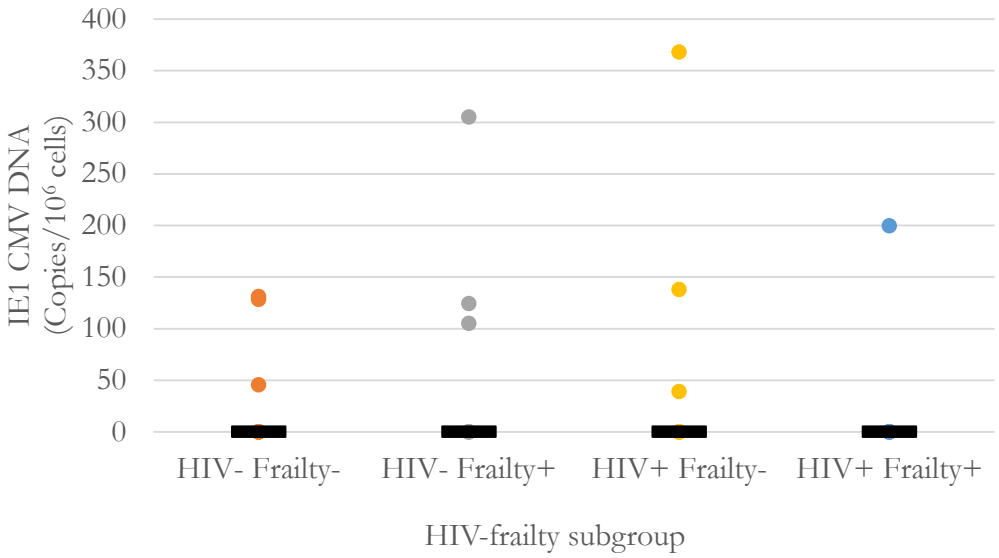


Table 3.5. Median Concentrations of IE1 DNA Detected in Each HIV-Frailty Subgroup

HIV serostatus	Frailty status	Median IE1 DNA detected (copies/10^6 cells)	IQR
HIV−	−	ND	0 – 45.5
	+	ND	0 – 109.9
HIV+	−	ND	0 – 38.9
	+	ND	0 – 0

Frailty− = negative for Fried frailty phenotype; frailty+ = positive for Fried frailty phenotype; ND= Not Detected; IQR= interquartile range

Table 3.6. P- values for Comparisons Between HIV-Frailty Subgroups for IE1 DNA Detected

	HIV− frailty+	HIV+ frailty−	HIV+ frailty+
HIV− frailty−	0.5	0.7	0.8
HIV− frailty+		0.5	0.5
HIV+ frailty−			0.1

b.

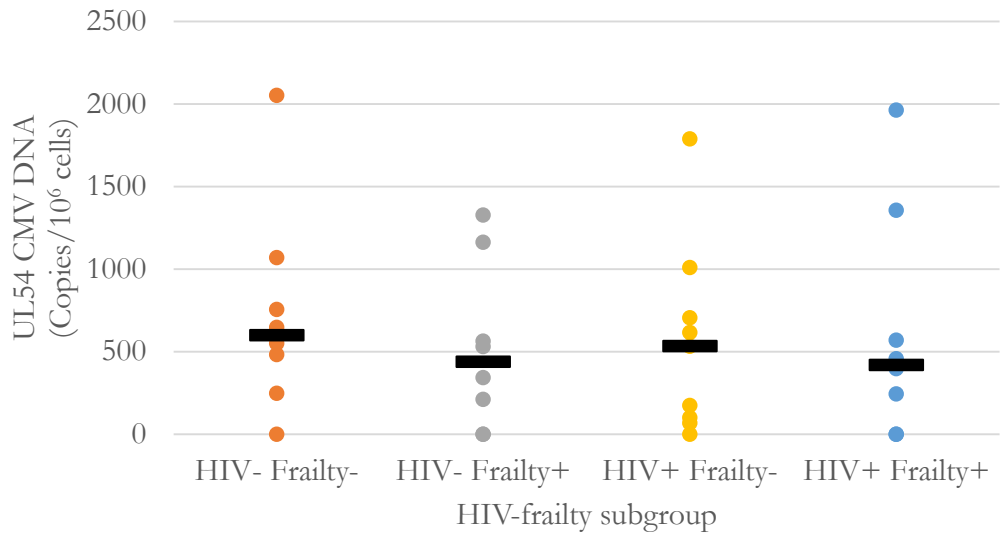


Table 3.7. Median Concentrations of UL54 DNA Detected in Each HIV-Frailty Subgroup

HIV serostatus	Frailty status	Median UL54 DNA detected (copies/10^6 cells)	IQR
HIV−	−	599	423.4 – 834.2
	+	437	158.4 – 712.5
HIV+	−	532.6	100.6 – 705.2
	+	418	60.8 – 540.8

Frailty− = negative for Fried frailty phenotype; frailty+ = positive for Fried frailty phenotype; IQR= interquartile range

Table 3.8. P- values for Comparisons Between HIV-Frailty Subgroups for UL54 DNA Detected

	HIV− frailty+	HIV+ frailty−	HIV+ frailty+
HIV− frailty−	0.79	0.81	0.56
HIV− frailty+		0.89	0.97
HIV+ frailty−			0.60

c.

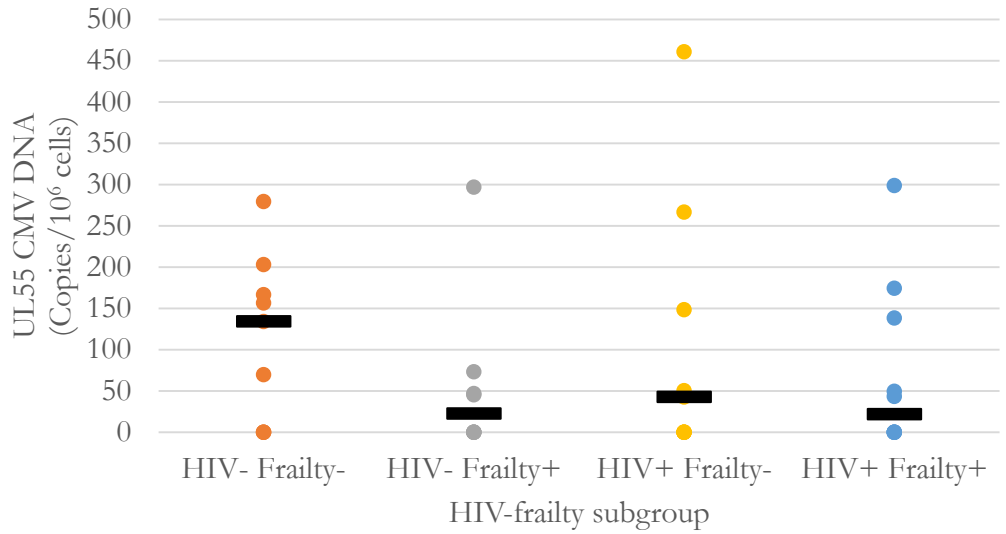


Table 3.9. Median Concentrations of UL55 DNA Detected in Each HIV-Frailty Subgroup

HIV serostatus	Frailty status	Median UL55 DNA detected (copies/10^6 cells)	IQR
HIV−	−	134.1	0 – 166.7
	+	22.6	0 – 53.5
HIV+	−	42.6	0 – 148.4
	+	21.7	0 – 116.1

Frailty− = negative for Fried frailty phenotype; frailty+ = positive for Fried frailty phenotype; IQR= interquartile range

Table 3.10. P-values for Comparisons Between HIV-Frailty Subgroups for UL55 DNA Detected

	HIV− frailty+	HIV+ frailty−	HIV+ frailty+
HIV− frailty−	0.3	0.9	0.5
HIV− frailty+		0.4	0.6
HIV+ frailty−			0.3

d.

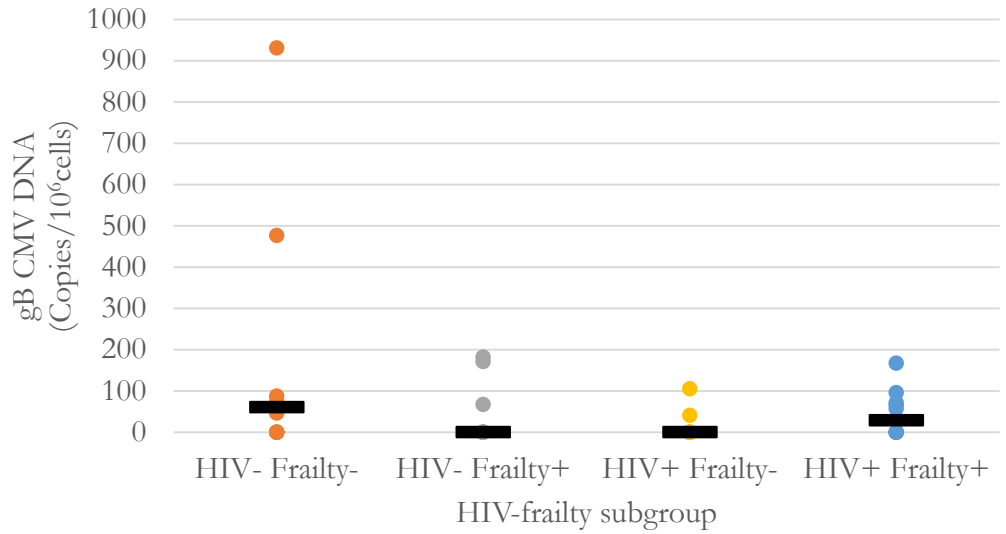


Table 3.11. Median gB DNA Detected in Each HIV-Frailty Subgroup

HIV serostatus	Frailty status	Median gB DNA detected (copies/10^6 cells)	IQR
HIV−	−	60.4	0 – 184.9
	+	ND	0 – 93.1
HIV+	−	ND	0 – 0
	+	28.71	0 – 69.9

Frailty− = negative for Fried frailty phenotype; frailty+ = positive for Fried frailty phenotype; ND= Not Detected

Table 3.12. P-values for Comparisons Between HIV-Frailty Subgroups for gB DNA Detected

	HIV− frailty+	HIV+ frailty−	HIV+ frailty+
HIV− frailty−	0.4	0.1	0.5
HIV− frailty+		0.5	1.00
HIV+ frailty−			0.3

3. The Relationship Between CMV DNA Levels and T Cell Responses to CMV Peptides

Having detected the amount of each CMV DNA, the donors were dichotomized into two groups: the top 25% of donors were considered those in whom the DNA was detected and the lower 75% of donors those in whom it was not. The number of T cells responding to a CMV peptide was compared between these two groups. This analysis was further stratified by the type of T cell responding to the CMV peptide (CD4 or CD8), by the cytokine produced by the T cell (IFNγ, TNF, or IL-2), and finally, by the CMV peptide that stimulated the T cell response. A Mann-Whitney U test was used to determine significance of differences in the T cell responses between those donors with and without detectable CMV DNA.

Some donors with detectable CMV DNA had T cell responses to CMV peptides. These responses varied depending on which CMV DNA was detected, which cells were responding to the CMV peptide, which cytokine was produced, and which peptide was stimulating the response. In addition to the T cell responses seen when CMV DNA was detected, some donors with undetectable CMV DNA had T cell responses to CMV peptide stimulation.

This section will first describe the T cell response in donors with detectable DNA, followed by the response seen in donors without detectable DNA. Next, it will present the effect that the presence of detectable CMV DNA had on the T cell response to CMV in HIV infection and in frailty.

i. T Cell Responses to CMV Peptide Stimulation in Donors When CMV DNA Was Detected

Donors with detectable CMV DNA had higher proportion of CMV-responsive T cells than donors with undetectable CMV DNA. This difference between the donor groups was significant depending on which cell responded by producing which cytokine. For example, donors with detectable UL54 DNA had a higher frequency of CD4 and CD8 T cells producing TNF in response to CMV stimulation than donors with undetectable UL54 DNA (Table 3.14). Another example was donors with detectable UL55 DNA, who had a greater number of CD4 cells that produced IL-2 in response to CMV peptide stimulation compared to donors with undetectable UL55 DNA (Table 3.15). In addition to the significant difference in the number of CD4 cells producing IL-2 in response to a variety of CMV peptides in donors with UL55 DNA detected, the total number of CD4 cells producing IL-2 was also higher in these donors compared to donors with undetectable UL55 DNA.

ii. T Cell Responses to CMV Peptide Stimulation in Donors When No CMV DNA Was Detected

Some donors had higher CMV-specific T cell responses when no CMV DNA was detected, compared to donors with detectable CMV DNA. For example, donors with undetectable IE1 DNA had a higher number of CMV-specific T cells producing a variety of cytokines, compared to donors with detectable IE1 DNA (Table 3.13). The number of CD4 and CD8 cells producing IFNγ, IL-2, and TNF were all higher in donors with undetectable IE1 DNA compared to donors with detectable IE1 DNA. Donors with other undetectable CMV DNAs, such as UL55 and UL54, had T cell responses to CMV that differed from the responses previously mentioned in donors with detectable amounts of these DNA. CD4 T cells showed higher IFNγ responses while CD8 T cells showed higher IFNγ, IL-2, and TNF responses in donors without UL55 DNA (Table 3.15). Other than showing the complexity of the T cell responses to CMV, these results show how detectable, or undetectable CMV DNA can affect these responses.

iii. The Effect of the Detection of CMV DNA on the T Cell Response to Stimulation Using a Related Peptide

If the CMV DNA is having an effect on the T cell response, it would be important to determine the importance of the specific CMV peptide used to stimulate the T cell response. Therefore, we studied whether the T cell response was different when it was stimulated by a CMV peptide that was closely related to the CMV DNA detected (Table 3.20). The number of CD4 T cells that produced TNF in response to stimulation by UL55 peptide was significantly different between HIV− frailty+ donors with detectable gB DNA or HIV+

frailty− donors with detectable UL55 DNA, compared to donors with either DNA undetectable (Table 3.19).

In contrast, in one subgroup of donors (HIV+ frailty+), the number of CD8 T cells that produced either IFNγ (Table 3.17) or TNF (Table 3.19) in response to stimulation by the UL123 peptide was significantly different between donors with undetectable IE1 DNA, compared to those donors with detectable IE1 DNA (Table 3.20). Together these results suggest a link between the peptide stimulating the T cell response, the T cell response to the stimulation, and detectable or undetectable CMV DNA.

iv. The Effect of Detectable CMV DNA on T Cell Responses to CMV in HIV and frailty

Having determined that detected CMV DNA may have an effect on T cell responses to CMV, the next aim of the project was to see if these effects were associated with HIV status and/or frailty status. Overall, the detection of CMV DNA was indeed associated with the T cell responses in donors, and this effect seemed to be unrelated to either HIV serostatus or frailty status on their own. However, when the results were further stratified into subgroups by HIV and frailty status, significant differences in T cell responses could be seen between donors with detectable DNA and those with undetectable DNA.

HIV+ frailty− donors had a higher frequency of CD4-responsive cells producing IL-2 when gB DNA was detected and also when UL55 DNA was detected, compared to donors with either DNA undetectable (Table 3.18). The fact that the gB probe and the UL55 primer each independently detected CMV DNA in donors, and that significant differences were

seen in the T cell response between these donors who had detectable CMV DNA and those donors without detectable CMV DNA, strengthens the results.

In HIV− frailty+ donors, there was a higher frequency of CD8 CMV-responsive cells producing IFNγ when gB DNA was detected and also when UL54 DNA was detected, compared to donors with either DNA undetectable (Table 3.17). This demonstrated a relationship between the detection of CMV DNA and the T cell responses in frail donors who were HIV−.

Significant differences in the same T cell response were seen in donors who were positive for multiple CMV DNA compared to those with undetectable CMV DNA, suggesting that the detection of CMV DNA may be linked to specific T cell responses to CMV.

v. Significant Differences in the T Cell Responses to CMV Between the HIV-Frailty Subgroups, Regardless of the Detection of CMV DNA

To test if there were significant differences in the T cell responses to CMV between the HIV-frailty subgroups, we compared the T cell responses to peptide stimulation in each HIV-frailty subgroup. Significant differences were seen between the HIV-frailty subgroups. The majority of significant differences in T cell responses to CMV stimulation were present in donors with undetectable CMV DNA. For example, the proportion of CD4 CMV-responsive T cells and the CD8 CMV-responsive T cells producing IFNγ showed significant differences between the HIV-frailty subgroups (Table 3.21).

A potential link between gB DNA and CD4 cells producing IL-2 (mentioned earlier) was supported by finding of significant differences in the frequency of these cells between the HIV-frailty subgroups when gB DNA was not detected in donors (Table 3.22).

Also, there were significant differences between the T cell responses of the HIV-frailty subgroups to specific CMV peptides. For example, stimulation of CD4 cells with the CMV peptide UL82 resulted in a significant difference in the T cell IFNγ response in the HIV-frailty subgroups when any of the CMV DNAs (i.e., IE1, UL54, UL55, and gB) were not detected (Table 3.21). This analysis helps identify which peptides have effects on the T cell responses in each HIV-frailty subgroup.

Table 3.13. Significant Differences in the Frequency of T Cell Responses to CMV Peptides Between Donors with Detectable IE1 DNA and Those with Undetectable IE1 DNA, in the Entire Study Population

Cells	Cytokine Response	Peptide stimulation	Donors with undetectable IE1 DNA (n=27)	Donors with detectable IE1 DNA (n=9)	P value
CD4	IFNγ	UL32b	**0.01**	0	0.06
		UL99	**0.03**	0.01	0.02
		UL122	**0.03**	0	0.004
	IL-2	UL36	**0.06**	0.01	0.007
		UL83	**0.1**	0.02	0.007
		UL99	**0.03**	0	0.01
		UL122	**0.03**	0.01	0.02
	TNF	UL32b	**0.03**	0	0.005
		UL122	**0.03**	0	0.03
CD8	IFNγ	UL123	**1.25**	0.25	0.01
		UL151	**0.03**	0	0.05
	IL-2	UL36	**0.05**	0	0.01
		UL94	**0.01**	0	0.03
		UL123	**0.08**	0.01	0.005
		UL153	**0.01**	0	0.04
		US3	**0.01**	0	0.03
	TNF	UL123	**1.02**	0.15	0.007
		UL151	**0.03**	0	0.05

Data are the median percentages of CD4 or CD8 T cell responses to CMV stimulation
Bold = the higher T cell response between donors with detectable or with undetectable CMV DNA.

Table 3.14. Significant Differences in the Frequency of T Cell Responses to CMV Peptides Between Donors with Detectable UL54 DNA and Those with Undetectable UL54 DNA, in the Entire Study Population

Cells	Cytokine Response	Peptide stimulation	Donors with undetectable UL54 DNA (n=27)	Donors with detectable UL54 DNA (n=9)	P value
CD4	IFNγ	UL32a	**0.11**	0	0.008
		UL48-b	**0.01**	0	0.05
		UL55-a	**0.07**	0.01	0.02
		UL99	**0.03**	0	0.02
		UL123	**0.03**	0	0.009
		US24	**0.01**	0	0.02
	IL-2	UL86-b	0	**0.09**	0.06
	TNF	UL28	0.01	**0.06**	0.04
		UL48 pool	0.02	**0.05**	0.03
		UL86-b	0.01	**0.2**	0.02
CD8	IFNγ	UL48-b	**0.02**	0	0.04
	TNF	UL48 pool	0.01	**0.14**	0.005
		UL48d	0	**0.08**	0.001
		UL55a	0.01	**0.1**	0.01
		UL122	0.04	**0.11**	0.06

Data are the median percentages of CD4 or CD8 T cell responses to CMV stimulation
Bold = the higher T cell response between donors with detectable or with undetectable CMV DNA.

Table 3.15. Significant Differences in the Frequency of T Cell Responses to CMV Peptides Between Donors with Detectable UL55 DNA and Those with Undetectable UL55 DNA, in the Entire Study Population

Cells	Cytokine Response	Peptide stimulation	Donors with undetectable UL55 DNA (n=27)	Donors with detectable UL55 DNA (n=9)	P value
CD4	IFNγ	UL55-b	0.06	**0.53**	0.007
		UL82	**0.03**	0	0.02
		UL86-a	**0.06**	0	0.009
		UL103	**0.02**	0	0.05
	IL-2	UL48-a	0	**0.09**	0.02
		UL48-c	0	**0.05**	0.03
		UL55-b	0.03	**0.31**	0.02
		UL94	0	**0.07**	0.02
		US24	0	**0.01**	0.06
Total CD4	IL-2		0.94	**4.16**	0.046
CD8	IFNγ	UL32-a	**0.03**	0	0.008
		UL48-a	**0.01**	0	0.04
		UL48-c	**0.01**	0	0.04
		UL82	**0.03**	0	0.06
		UL86-b	**0.03**	0	0.03
	IL-2	UL28	**0.01**	0	0.03
		UL86-a	**0.01**	0	0.03
		UL86-b	**0.01**	0	0.02
		UL99	**0.01**	0	0.04
		UL151	**0.02**	0	0.05
		US29	**0.02**	0	0.06
		US32	**0.09**	0.01	0.05
	TNF	UL32-a	**0.03**	0	0.007
CD8+CD28-CD27+ CD45RA+			**4.6**	1.8	0.06

Data are the median percentages of CD4 or CD8 T cell responses to CMV stimulation

Bold = the higher T cell response between donors with detectable or with undetectable CMV DNA.

Total CD4 is sum of all CD4 T cell responses to peptide stimulation

Table 3.16. Significant Differences in the Frequency of T Cell Responses to CMV Peptides Between Donors with Detectable gB DNA and Those with Undetectable gB DNA, in the Entire Study Population

Cells	Cytokine Response	Peptide Stimulation	Donors with undetectable gB DNA (n=27)	Donors with detectable gB DNA(n=9)	P value
CD8	TNF	UL103	0	0	0.035
		UL153	0	**0.5**	0.021
	IFNγ	UL153	0	**0.8**	0.01
CD4+CD45RA+ CD27+			**20.3**	5.8	0.036
CD8+CD28-CD45RA-CD27+			**2.3**	0.5	0.036
CD8+CD28-CD45RAmid CD27+			**2.2**	0.5	0.007

Data are the median percentages of CD4 or CD8 T cell responses to CMV stimulation
Bold = the higher T cell response between donors with detectable or with undetectable CMV DNA.

Table 3.17. Significant Differences in The Frequency of T Cells Producing IFNγ in Response to CMV Peptides Between Donors with Detectable CMV DNA and Those with Undetectable CMV DNA by HIV-Frailty Subgroup

HIV serostatus	Frailty status	Cell	CMV gene detected	Peptide stimulation	Donors with undetectable CMV DNA	Donors with detectable CMV DNA	P value
HIV−	Frail−	CD4	UL54	UL123	0	**0**	0.043
				UL48 pool	0	**0.2**	0.036
				US24	0	0	0.042
			UL55	UL32-b	**0.1**	0	0.032
				UL86-a	**0.1**	0	0.013
		CD8	IE1	UL151	**0.3**	0	0.043
				UL28	**0.5**	0	0.018
			UL54	US29	**0.7**	0	0.043
			UL55	US32	**1.4**	0.2	0.014
	Frail+	CD4	gB	UL48 pool	0	0	0.043
				UL55-a	0	**0.5**	0.044
				UL82	**0.1**	0	0.043
			IE1	UL55-a	0	**0.2**	0.024
		CD8	gB	UL153	0	**1.3**	0.032
				US29	0	**2.2**	0.043
				US32	0.1	**2.7**	0.044
			UL54	UL123	0.2	**1.3**	0.046
				UL48 pool	0	**0.3**	0.044
				UL48-d	0	**0.2**	0.043
				UL48-e	0	**0.1**	0.043
				UL55-a	0	**0.1**	0.044
HIV+	Frail−	CD4	UL55	UL122	0	**0.4**	0.036
				UL48 pool	0	**0.2**	0.032
				UL48-d	0	**0.3**	0.035
		CD8	IE1	UL123	**2.3**	0	0.04
			UL54	US3	0	**0.7**	0.025
			UL55	UL32-a	**0.1**	0	0.036
	Frail+	CD4	gB	UL48-b	0	**0.1**	0.05
			UL54	SEB	**5.9**	3.4	0.037
		CD8	UL54	UL55-a	**0.1**	0	0.047
				US32	**0.3**	0	0.036

Data are the median percentages of CD4 or CD8 T cell responses to CMV stimulation

Bold = the higher T cell response between donors with detectable or with undetectable CMV DNA.

SEB= Staphylococcal Enterotoxin B

Table 3.18. Significant Differences in the Frequency of T Cells Producing IL-2 in Response to CMV Peptides Between Donors with Detectable CMV DNA and Those with Undetectable CMV DNA by HIV-Frailty Subgroup

HIV serostatus	Frailty status	Cell	CMV detected	Peptide stimulation	Donors without detectable CMV	Donors with detectable CMV	P value
HIV−	Frail−	CD4	IE1	UL32-a	**0.04**	0	0.042
			UL54	UL48 pool	0	**0.11**	0.036
		CD8	gB	SEB	1.98	**4.89**	0.043
			IE1	UL151	**0.04**	0	0.042
			UL54	UL48-b	0	**0.06**	0.048
				UL48-c	0	**0.28**	0.035
			UL55	UL28	**0.15**	0	0.032
				UL32-a	**0.02**	0	0.031
				UL82	**0.01**	0	0.031
				UL94	**0.03**	0	0.032
				UL99	**0.02**	0	0.031
				UL103	**0.03**	0	0.047
				UL153	**0.03**	0	0.01
				US29	**0.12**	0	0.017
				US32	**0.22**	0	0.014
	Frail+	CD4	gB	UL32-a	**0.07**	0	0.046
				UL86-a	**0.09**	0	0.043
				UL122	**0.03**	0	0.043
			IE1	UL55-a	0.01	**0.06**	0.024
				UL122	**0.04**	0	0.035
			UL54	US3	0	**0.44**	0.022
		CD8	IE1	UL86-a	**0.04**	0	0.021
			UL54	UL83	**0.04**	0.01	0.043
HIV+	Frail−	CD4	gB	SEB	6.37	**18.9**	0.037
				UL86-b	0	**0.22**	0.026
				US32	0	**0.08**	0.019
			UL54	UL82	0	**0.04**	0.045
			UL55	UL36	0.02	**0.41**	0.036
				UL48 pool	0	**0.16**	0.015
				UL48-a	0	**0.1**	0.025
				UL48-b	0	**0.17**	0.024
				UL48-c	0	**0.08**	0.032
				UL94	0	**0.21**	0.046
				UL99	0.02	**0.13**	0.036
				UL103	0.02	**0.13**	0.054
				UL122	0	**0.17**	0.045
				Total	0.64	**4.24**	0.04
		CD8	UL54	UL55-a	0	**0.17**	0.032
				UL82	0	**0.33**	0.025
			UL55	UL94	0	**0.19**	0.025
	Frail+	CD4	UL55	UL32-b	0.01	**0.49**	0.034
		CD8	UL54	UL83	0.04	**0.42**	0.036

Data are the median percentages of CD4 or CD8 T cell responses to CMV stimulation
Bold = the higher T cell response between donors with or without detectable CMV DNA.
SEB= Staphylococcal Enterotoxin B

Table 3.19. Significant Differences in the Frequency of T Cells Producing TNF in Response to CMV Peptides Between Donors with Detectable CMV and Those with Undetectable CMV DNA by HIV-Frailty Subgroup

HIV serostatus	Frailty status	Cell	CMV gene detected	Peptide stimulation	Donors without detectable CMV	Donors with detectable CMV	P value
HIV−	Frail−	CD4	gB	UL48-a	**0.04**	0	0.047
				UL48-e	**0.02**	0	0.047
			UL54	UL122	**0.04**	0	0.043
			UL55	UL86-a	**0.04**	0	0.018
		CD8	gB	UL48-e	**0.03**	0	0.026
				UL86-a	0	**0.01**	0.047
				UL103	0	**0.1**	0.038
			IE1	UL32-a	**0.03**	0	0.043
				UL151	**0.11**	0	0.018
			UL54	UL32-b	0	**0.03**	0.034
				UL36	0	**0.05**	0.011
				UL48 pool	0.01	**0.14**	0.035
				UL48-a	0	**0.09**	0.015
				UL48-d	0	**0.1**	0.015
			UL55	UL28	**0.38**	0	0.032
				US29	**0.49**	0.01	0.049
				US32	**0.71**	0.03	0.014
	Frail+	CD4	gB	SEB	9.93	**18.17**	0.046
				UL55-a	0.03	**0.53**	0.043
			IE1	UL32-b	**0.04**	0.01	0.046
		CD8	UL54	UL48 pool	0.02	**0.27**	0.044
				UL48-d	0.01	**0.15**	0.04
				UL55-a	0.01	**0.11**	0.043
HIV+	Frail−	CD4	UL55	UL28	0.03	**0.31**	0.035
				UL48-d	0	**0.34**	0.032
				UL48-e	0	**0.09**	0.036
				UL55-b	0.06	**0.3**	0.055
				UL123	0.01	**0.21**	0.037
				UL151	0.01	**0.24**	0.052
		CD8	UL54	US3	0.01	**0.62**	0.038
			UL55	SEB	**23.6**	10.76	0.04
	Frail+	CD4	gB	SEB	7.04	**23.83**	0.037
			IE1	UL83	**0.26**	0	0.04
			UL54	SEB	**8.32**	1.93	0.037
		CD8	IE1	UL123	**1.41**	0.01	0.04
			UL54	SEB	**13.71**	8.13	0.037
				UL48-b	0	**0.05**	0.055
				UL55-b	0	**0.12**	0.047
				UL99	0	**0.33**	0.025
			UL55	UL103	0	**0.14**	0.031
				US24	**0.03**	0	0.033

Data are the median percentages of CD4 or CD8 T cell responses to CMV stimulation
Bold = the higher T cell response between donors with or without detectable CMV DNA.
SEB= Staphylococcal Enterotoxin B

Table 3.20. Significant Differences in T Cell Responses to Stimulation with CMV Peptide when the Related CMV DNA is Detected

This table shows which CMV DNA was detected or not detected, which peptide stimulated the T cell response, whether the significant response was in the whole cohort or which HIV frailty subgroup, which T cell population had the response and what the response was.

CMV DNA	DNA detected (+) or not detected (−)	Peptide stimulation	Whole cohort or HIV-frailty subgroup	T cell	Cytokine response
gB	−	UL55	HIV− frailty+	CD4	IFNγ
	+				TNF
UL55	+	UL55	Whole cohort	CD4	IFNγ
					IL-2
			HIV+ frailty−		TNF
IE1	−	UL122	Whole cohort	CD4	IFNγ
					IL-2
					TNF
		UL123		CD8	IFNγ
					IL-2
					TNF
			HIV+ frailty+		IFNγ
					TNF
		UL122	HIV− frailty+	CD4	IL-2

UL123 = IE1; UL122 = IE2

Table 3.21. Differences in T Cell IFNγ Responses to CMV Peptide Stimulation Between HIV-Frailty Subgroups, for Donors without and with Detectable CMV DNA

Cell	Peptide stim	Donors without detectable IE1 DNA	Donors with detectable IE1 DNA	Donors without detectable UL54 DNA	Donors with detectable UL54 DNA	Donors without detectable UL55 DNA	Donors with detectable UL55 DNA	Donors without detectable gB DNA	Donors with detectable gB DNA
CD4	CMV antigen	0.071	0.126	0.115	0.086	**0.021**	0.101	**0.044**	0.237
	SEB	0.173	0.223	0.269	0.057	**0.021**	0.753	**0.048**	0.423
	UL103	0.814	0.166	0.969	0.411	0.618	0.049	0.92	0.462
	UL32-b	**0.025**	0.365	0.016	0.766	0.093	0.181	**0.044**	0.782
	UL82	**0.004**	0.513	**0.03**	0.814	**0.029**	0.726	**0.006**	0.3
	UL86-a	**0.037**	0.801	0.28	0.202	0.059	0.5	**0.024**	0.703
	UL86-b	**0.018**	0.626	0.213	0.254	**0.026**	0.536	**0.018**	0.411
CD8	UL122	0.092	0.198	0.134	0.257	**0.043**	0.818	0.213	0.323
	UL28	**0.007**	0.083	0.059	0.501	**0.015**	0.557	**0.006**	0.356
	UL32-a	0.591	0.183	0.772	0.157	0.33	**0.046**	0.481	0.556
	UL36	0.063	0.9	0.08	0.553	**0.04**	0.934	0.553	0.102
	UL48-b	0.927	**0.054**	0.872	**0.049**	0.654	0.176	0.829	0.436
	US3	0.173	0.709	**0.039**	0.128	0.494	0.266	0.319	0.324

Differences in the CD4 or CD8 T cell IFNγ response to CMV peptides in donors either without detectable or with detectable CMV DNA between HIV-frailty subgroups. Differences were measured by a Kruskal-Wallis test.

Bold = significant difference (p value <0.05)

Table 3.22. Differences in T Cell IL-2 Responses to CMV Peptide Stimulation Between HIV-Frailty Subgroups, for Donors without and with Detectable CMV DNA

Cell	Peptide stim	Donors without detectable IE1 DNA	Donors with detectable IE1 DNA	Donors without detectable UL54 DNA	Donors with detectable UL54 DNA	Donors without detectable UL55 DNA	Donors with detectable UL55 DNA	Donors without detectable gB DNA	Donors with detectable gB DNA
CD4	SEB	0.062	0.232	**0.022**	0.228	**0.033**	0.423	**0.02**	0.195
	UL32-a	0.082	0.279	0.169	0.984	0.18	0.94	**0.047**	0.884
	UL55-a	0.241	0.39	0.431	0.799	0.517	0.847	**0.039**	0.459
	UL86-b	0.079	0.994	0.389	0.261	0.066	0.611	**0.036**	0.305
CD8	UL86-a	0.305	**0.049**	0.717	0.115	0.791	0.321	0.504	0.172
	UL94	0.848	0.321	0.892	0.774	0.328	**0.049**	0.989	0.266

Differences in the CD4 or CD8 T cell IL-2 response to CMV peptides in donors either without detectable or with detectable CMV DNA between HIV-frailty subgroups. Differences were measured by a Kruskal-Wallis test.

Bold = significant difference (p value <0.05)

Table 3.23. Differences in T Cell TNF Responses to CMV Peptide Stimulation Between HIV-Frailty Subgroups, for Donors without and with Detectable CMV DNA

Cell	Peptide stim	Donors without detectable IE1 DNA	Donors with detectable IE1 DNA	Donors without detectable UL54 DNA	Donors with detectable UL54 DNA	Donors without detectable UL55 DNA	Donors with detectable UL55 DNA	Donors with undetectable gB DNA	Donors with detectable gB DNA
CD4	UL86-a	**0.046**	0.718	0.26	0.2	0.367	0.118	0.397	0.338
	UL86-b	0.106	0.8	0.681	0.261	0.196	0.301	**0.045**	0.606
	US24	0.1	0.673	0.121	**0.049**	0.374	0.124	0.223	0.61
	US29	0.419	0.671	0.99	**0.049**	0.744	0.068	0.786	0.471
CD8	UL28	0.055	0.384	**0.046**	0.929	0.086	**0.048**	0.093	0.718
	UL36	0.27	0.886	**0.049**	0.212	0.192	0.744	0.526	0.348
	UL86-b	**0.023**	0.064	**0.003**	0.49	**0.035**	**0.054**	**0.021**	0.199
	UL99	0.117	0.383	**0.032**	0.383	0.62	0.088	0.205	0.45
	US3	**0.049**	0.126	**0.009**	0.205	**0.049**	0.4	0.093	0.425

Differences in the CD4 or CD8 T cell TNF response to CMV peptides in donors either with undetectable or with detectable CMV DNA between HIV-frailty subgroups. Differences were measured by a Kruskal-Wallis test.

Bold = significant difference (p value <0.05)

IV. Discussion

A. Importance of the Project

The introduction of HAART has helped people live longer with HIV infection, but has led to a rise in HANA conditions, such as frailty. Chronic inflammation is a hallmark of HIV infection and frailty, with common serological inflammatory markers TNF, IL-6, and CRP elevated in both conditions. Although the inflammation is reduced in HIV+ people receiving HAART compared to HIV+ people not receiving HAART, chronic inflammation persists and can cause severe cell and tissue damage. A common etiology for the chronic inflammation present in both HIV infection and frailty could be a persistent viral infection, such as CMV. The prevalence of CMV in nearly all HIV+ and frail people and the ability of the virus to elicit a strong cellular immune response, make CMV a good candidate for causing, at least in part, the chronic inflammation. Understanding the causes of this chronic inflammation could lead to therapies that reduce or prevent chronic inflammation. Such therapies would be a significant benefit, not just to the growing number of people living longer with HIV, but also to the HIV− elderly population and people with other conditions (such as atherosclerosis and cancer) where inflammation is a significant problem.

This project built on previous studies, including the work of Margolick *et al.* (8) (discussed in more detail in the Introduction), which reported that the increased number of CMV-responsive T cells could influence the chronic inflammation seen in both HIV-infected and HIV− men. Although the CD4 and CD8 T cells from all of the donors tested in the study

by Margolick *et al.* (8) responded to at least some of the CMV peptides tested, the presence of CMV in these donors was not tested. This left unanswered the important question of whether the amount of CMV present accounts for the differences in the frequency of CMV-responsive T cells. By quantifying the CMV DNA and comparing it with known T cell response data, this project aimed to improve our understanding of the role CMV plays in the pathogenesis of inflammation.

B. Validating the ddPCR Assay

1. Assessing the Sensitivity of the ddPCR Assay

After an initial lytic phase of infection, CMV enters a latent phase with minimal replication of its viral genes. It has yet to be fully established which cells the latent CMV resides in. The lack of replicating virus and the very small number of infected cells meant a highly sensitive assay would be needed. In ddPCR, the DNA in a specimen is separated into 20,000 nanoliter-sized droplets, so that each droplet gets approximately one copy of the template DNA. By separating the template DNA into so many droplets, non-specific background DNA is reduced, which improves the PCR conditions. This helps make the ddPCR assay more sensitive than other PCR assays (e.g., RT-PCR) at detecting rare DNAs. The ability of ddPCR to provide an absolute quantitation of the DNA concentration without the need for an external standard, as discussed in the Methods, also makes the ddPCR assay a valuable method to quantify rare DNAs.

When the project was started, ddPCR was a new technique that needed validation to determine how sensitive and accurate it was before it could be reliably used on the samples.

A recently published paper that used ddPCR to measure CMV (119) was used as the starting point for developing the assay.

The availability of the IE1 primers that previously had been used by Dr. Leng to detect CMV using nested PCR (124) and the importance of IE1 in viral replication made IE1 a good target to start with to establish how effective the ddPCR was at detecting CMV DNA. Using a series of 10-fold serial dilutions of genomic AD169 CMV that was newly purchased, ddPCR could detect 1 copy of CMV DNA (Figure 3.1). Using HFFs infected with CMV at multiple MOIs, CMV DNA could be detected by ddPCR at low copy number (Figure 3.2). The sensitivity of the ddPCR assay to detect CMV at low levels led to the testing of PBMC DNA for CMV DNA. However, it was more difficult to detect CMV DNA in PBMC when it was unknown if CMV DNA was present, and even if present was likely to be at very low levels. The challenge was to distinguish low levels of CMV DNA from nonspecific binding of the primers or the probe, that lead to fluorescence which was not due to binding to CMV DNA. To address this challenge, the specificity of the primers and the probe had to be determined.

2. Determining the Specificity of the Primers and Probes Used in the ddPCR Assay

The presence of positive fluorescent droplets in known CMV− samples, after the background had been accounted for, raised questions over the accuracy of the positive signal (Figure 3.6). CMV DNA in PBMC is so rare that it may be represented by only one or two droplets. The verity of a positive droplet in a sample could be undermined by any false-

positive droplets detected in the negative controls. The importance of having negative controls that were reliably clear of any positive droplets meant much time was spent on ensuring the cleanest possible laboratory environment to avoid any possibility of contamination of the samples.

Testing CMV− samples helped determine the specificity of the primers and probes used. These samples were either from donors who had been confirmed to be CMV− by an ELISA or nested PCR, such as PBMC from a CMV− leukopak, or mouse tail DNA which cannot be infected with human CMV. While the background levels varied between the primers and in each CMV− sample, the gB probe consistently had negligible background levels (Figure 3.7). Using known CMV− samples as negative controls in subsequent experiments in addition to the NTC, served as a better negative control than the NTC alone. The background levels of the primers had to be accounted for and subtracted from any positive signal measured by the primers. However, using multiple negative controls provided the best gauge of the background levels and increased the likelihood that the positive droplets truly were due to the detection of CMV DNA.

3. Determining the Optimal Amount of Genomic DNA in Each Well

To maximize the chance of detecting CMV DNA, the amount of DNA that could be loaded in each well was determined. This amount was determined to be 50 ng, based on the finding that at this amount CMV DNA could be detected while preserving good separation between positive and negative droplets (Figure 3.4). This separation is important as the negative droplets must be low enough to clearly identify positive fluorescent droplets. Higher

amounts of DNA were tested, but the separation between the positive and negative droplets was poorer compared to the 50 ng.

4. Determining which Primers to Use in the ddPCR Assay

As previously mentioned, IE1 was a good primer to start with as it had been used previously by Dr. Leng to detect CMV DNA by nested PCR (118). In addition to IE1, other primers were selected for study through literature searches or by their significance in CMV replication. UL54 and UL55 primers were selected for study based on a paper that compared multiple primers to different CMV genes, and these primers were determined to be the most sensitive and specific (123). US28 primers were tested as UL28 is an important CMV gene expressed both during the viral lytic replication and during latency. In initial experiments, the US28 primer detected high levels of CMV DNA. However, testing the CMV+ and CMV− samples showed that detection of US28 DNA was not specific to CMV+ cells, because there was no difference in detection between the CMV− and the CMV+ donors (Figure 3.6).

It would be worth designing new primers or probes that are more specific to determine the amount of US28 DNA present. The ability of the virus to remain latent the majority of the time in an infected person, makes it important to study those few genes that are expressed by the virus during latency, such as US28. This may provide insight into the role that these genes play in modulating the immune response to CMV.

5. Incorporating the gB Probe into the ddPCR Assay

The gB probe was incorporated into the assay as it had been used by another collaborator to detect CMV DNA (119). It used a different method of fluorescently tagging the target DNA that was hypothesized to be more specific and reduce the background levels. As previously discussed in the Methods, the probe binds to a target sequence already amplified by gene specific primers and during the extension phase the reporter attached to the probe is cleaved, allowing the reporter to fluoresce. The gB probe tagged with FAM allowed the use of a HEX-tagged RPP30 probe in the same well. Having the RPP30 in the same well as the CMV improved the accuracy of determining the sample cell number. It also allowed more samples to be tested on a plate.

As previously discussed, the gB probe had negligible background levels in the CMV− samples, indicating that the specificity of the probe was higher than that of the primers (Figure 3.7d). When tested in the same sample, the amount of gB DNA detected closely matched the amount of UL55 DNA detected (Figure 3.8), validating the results obtained by both the probe and the primers. Although the background levels always needed to be subtracted from the CMV levels detected by the primers, which was not a problem with the gB probe, the comparable levels of CMV DNA detected by the gB probe and the primers, especially at high copy numbers of CMV DNA, provides confidence in the results obtained by both the probe and the primers.

6. Confidence in the Results of the ddPCR Assay

The accuracy of the ddPCR results was further supported by the use of nested PCR to confirm the ddPCR results (Figure 3.3). Nested PCR was performed using the same IE1 primers used in the ddPCR assay for the second round of nested PCR reactions. CMV+ samples positive by ddPCR were also positive for IE1 by nested PCR, while the CMV− sample was negative by both methods.

Multiple CMV primers and the gB probe showed little intra-sample variation, which helped corroborate each other's results. In PBMC and semen, nearly all samples were positive for more than one CMV primer or probe. All samples that were positive for the gB probe were positive for at least one CMV primer. The amount of CMV DNA detected was also very similar between the primers and the gB probe, especially at higher concentrations of CMV DNA. In the semen samples the median amount of CMV DNA detected by IE1, UL54, UL55, and gB probe was 1.13, 1.09, 1.38 and 1.15×10^4 copies/10^6 cells respectively. The variation seen in the amount of CMV genes detected could be due to differences in sensitivity of each primer. However, the amount of CMV DNA detected by the UL55 primer strongly correlated with the amount of CMV DNA detected by the gB probe (Figure 8). As the UL55 and the gB probe are targeting the same gene, these results corroborate each other. This provides confidence in the results of the assay to detect CMV DNA.

The variation in the amount of CMV detected may also be due to the absence of the viral genes from the cell. It is possible that the donors are infected with different strains of the virus, or that during infection of the cell or during latency portions of the viral genome may be lost. For example, the commonly used laboratory CMV strain AD169 has a section of its

genome missing. The missing section encodes 19 ORFs that are present in clinical isolates, which prevent AD169 from replicating in cell types that are permissive for replication by clinical strains (125). While many ORFs are highly conserved between CMV strains, there is also substantial variability in the ORF sequences between CMV strains (126). Different viral strains may be detected better by some primers than others, depending on the variation in the target gene and how sensitive and specific the primer is. The consistently low background of the gB probe allowed the detection of droplet clusters of different fluorescent intensities in some samples, which may indicate variants of CMV.

To investigate the prevalence of different clinical CMV strains, a study of 53 immunocompetent women who had recently seroconverted to CMV identified a single clinical strain in 51/53 women based on UL55 sequencing (127). Over the 3 years that these women were followed, the same strain was present in each isolate tested. None showed signs of acquisition of new strains or sequence changes. Five subtypes of gB alone were identified in this study, and when this was combined with sequencing of 2 additional loci (UL144 and UL146), 32/53 women had their own unique strain of CMV (127). While the presence of multiple strains is uncommon in immunocompetent individuals, multiple strains have been identified in people with AIDS (128) and transplant recipients (129). Geographic and demographic variation can affect the gB genotype distribution (130); however, different genotypes have not been shown to have an effect on the clinical disease (131). Sequencing the CMV DNA from donors would reveal differences in the CMV strains that may help explain the variety of T cell responses seen to CMV DNA.

C. The Relationship Between CMV DNA and HIV and Frailty

This work aimed to take advantage of a small but well-characterized cohort of donors in the SHARE study (8). The frequency of CMV-responsive T cells was measured in PBMC. All donors had both CD4 and CD8 T cells responding to CMV peptide stimulation, regardless of HIV serostatus or frailty status. Levels of responsive T cells correlated with certain HIV and frailty subgroups. Higher levels of CD4+ T cells producing IL-2 in response to CMV stimulation predicted the onset of frailty in HIV− frailty− donors but not in HIV+ frailty− donors (8). This project aimed to build on that study by understanding how the presence of CMV DNA is related to the T cell responses to CMV.

As detailed in the Results, the present study showed that there were no significant differences in the amount of CMV DNA detected in PBMC between HIV+ and HIV− donors (8) (Figure 3.10a) or between frail and non-frail donors (Figure 3.10b). There were also no significant differences in the amount of CMV DNA detected in PBMC by any of the primers or the probe between the 4 subgroups: HIV− frailty−, HIV+ frailty−, HIV− frailty+, HIV+ frailty+ (Figure 3.11a-d). The lack of significant differences in the amount of CMV DNA detected in PBMC between HIV+ and HIV−, frailty+ and frailty−, and in the HIV-frailty subgroups suggests that detection of CMV DNA is not related to HIV or frailty status. Although the differences between some of the groups were not significant using a p-value cutoff of 0.05, some subgroups did show differences that were close to being significant, such as the amount of UL55 DNA in frailty− compared to frailty+ (Table 3.4), or IE1 DNA between HIV+ frailty− donors and both the HIV− frailty− donors and the

HIV+ frailty+ donors (Table 3.6), and gB DNA between the HIV− frailty− and the HIV+ frailty+ donors (Table 3.12).

It is possible that the small sample size and the variation within the groups for each gene could explain why the differences seen were not significant. The magnitude of the differences varied greatly within the groups analyzed. In many groups studied, there were either a majority or at least a large number of donors who had no CMV DNA detected. This varied between the specific primers and the probe tested. The variability in the amount of CMV DNA in PBMC detected between donors of the same group may have resulted in significant differences between the groups being missed. If a type II error has occurred, this could be addressed by studying a larger sample size. Studying a larger sample size may help resolve whether significant differences present in the current cohort hold up when more people are included in the study. It may also clarify whether differences that were close to the 0.05 p-value cutoff were actually significant or not.

D. The Detection of CMV DNA Correlates with Specific CMV-Stimulated T Cell Responses

1. T Cell Responses to CMV Stimulation when DNA is Detected

The relationship between the detection of CMV DNA in donors and their T cell responses to CMV stimulation, was found in all HIV-frailty subgroups and was independent of HIV or frailty status. Although the detection of CMV DNA was not affected by frailty status when viewed at the whole cohort level, stratification of the cohort revealed significant differences not previously seen. This may indicate that while the detection of CMV DNA alone may

not be related to frailty, it may have an effect on specific cell populations in people with or without HIV infection. The detection of certain CMV DNAs, such as UL55, was associated with certain T cell responses that could be identified in the whole cohort and in specific subgroups, such as the HIV+ frailty− subgroup. The number of CD4 IL-2 producing cells was higher when UL55 DNA was detected, in the whole cohort and in HIV+ frailty− donors (Table 4.1). When UL55 DNA was detected, the number of CD4 cells producing IFNγ and TNF was also significantly higher. The detection of other CMV DNAs in PBMC from donors also showed significant differences in the number of CD4 and CD8 CMV-responsive cells. Donors in whom UL54 was detected had significantly higher number of CD8 T cells producing TNF in responses to CMV, than donors in whom it was not detected. This association was present when the whole cohort was studied and in multiple HIV-frailty subgroups (Table 4.1). These results showed that the detection of different CMV DNAs was linked to varied T cell responses to CMV stimulation.

The ability of different CMV genes to have varied effects on the T cell responses to CMV stimulation has not been reported. These different responses may be due to the generation of abortive CMV transcripts that stimulate a T cell response, or the activation of innate immune sensors that trigger multiple different signaling pathways resulting in different responses. These possibilities are discussed in more detail later in this section.

Table 4.8. Examples of Significant Differences T Cell Responses when CMV DNA is Detected

This table shows which CMV DNA was detected, whether the significant response was in the whole cohort or specific HIV-frailty subgroup, which T cell population had the response, and what the response was.

CMV DNA detected	Group or subgroup	T cell	Cytokine response
gB	HIV− frailty+	CD8	IFNγ
		CD4	TNF
	HIV+ frailty-		IL-2
UL55	HIV+ frailty-	CD4	IFNγ
	Whole cohort	Total CD4	IL-2
		CD4	
	HIV+ frailty-	Total CD4	IL-2
		CD4	
			TNF
UL54	Whole cohort	CD4	TNF
		CD8	
	HIV− frailty+	CD8	IFNγ
			TNF
	HIV− frailty-		
	HIV+ frailty+		

2. T Cell Responses when the Stimulating CMV Peptide is Related to the DNA Detected

T cell responses showed significant differences between donors with detectable CMV DNA and those donors without CMV DNA, when the CMV DNA detected and the peptide used to stimulate the T cell response were from the same gene (Table 3.20). Donors with detectable UL55 DNA had significantly higher numbers of CD4 cells producing IFNγ and

IL-2 in response to UL55 than donors with undetectable UL55 DNA (Table 3.15). HIV+ frailty− donors with detectable UL55 DNA had significantly higher numbers of CD4 cells producing TNF in response to UL55 than donors with undetectable UL55 DNA (Table 3.19). These data are supported by the data which showed that HIV− frailty+ donors with detectable gB DNA, had significantly higher numbers of CD4 cells producing TNF in response to UL55 than donors with undetectable gB DNA (Table 3.19). A significant difference between the HIV-frailty subgroups was seen in donors with undetectable gB DNA, who had a higher number of CD4 cells producing IL-2 in response to UL55 peptide stimulation (Table 3.22). UL55 primers and gB probe both target the UL55 gene, which encodes the gB protein (as previously discussed), suggesting a link between the detection of UL55 DNA and T cell responses to UL55.

3. T Cell Responses to CMV when CMV DNA is Not Detectable

While the detection of UL55 DNA was linked with the T cell responses to UL55, donors with undetectable IE1 DNA had significantly higher number of responsive-T cells to either IE1 (CMV gene UL123) or related IE2 (CMV gene UL122). Donors with undetectable IE1 DNA had significantly higher numbers of CD4 cells producing IFNγ, TNF, and IL-2 in response to UL122 than donors with detectable IE1 DNA (Table 3.13). Donors with undetectable IE1 DNA had significantly higher numbers of CD8 cells producing IFNγ and IL-2 in response to UL123 than donors with undetectable IE1 DNA (Table 3.13). IE1 and IE2 are related proteins with similar functions. However, it appears that UL122 (IE2) was associated with a response in CD4 cells while UL123 (IE1) was associated with a CD8 response. It is unclear how this may be occurring, though presentation of IE1 peptides on MHC-I or IE2 peptides on MHC-II would activate CD8 or CD4 T cells, respectively.

T cell responses to CMV when CMV DNA is not detected in PBMC may indicate that CMV DNA is present in other parts of the body, where it can stimulate T cells. This could explain how the T cells are being activated even though the DNA is not detected in the PBMC. To test this, cells from different locations in the body could be isolated, such as the lungs and the spleen where CMV has previously been found (132), and compared between individuals that have detectable CMV DNA and those with undetectable CMV DNA.

E. Possible Mechanisms by which CMV DNA Could Affect T Cell Responses

CMV DNA may influence CD4 and CD8 T cell responses by a variety of mechanisms. One way that CD4 and CD8 cells are activated is by specific antigens. This requires the expression of viral peptides that are then presented on either MHC-II or MHC-I to CD4 or CD8 cells, respectively. The transcription of the CMV DNA and translation into viral proteins could stimulate CD4 and CD8 cells. However, CMV infection in these donors is in a latent phase, resulting in the expression of very few viral proteins. It is possible that the virus starts replicating but undergoes abortive transcription and only small messenger RNAs (mRNAs) are made (133). These could be translated into the viral peptides that stimulate the host immune response. This could explain why T cells have significant responses to the UL55 peptide pool in the presence of UL55 DNA and why some people have up to 30% CMV-specific T cells. To test whether abortive transcription is occurring in cells latently infected with CMV, very low levels of CMV RNA would need to be measured, which would require a very sensitive assay. It is possible that ddPCR may be sensitive enough to detect

these abortive transcripts. Reverse transcriptase would be needed to convert RNA into cDNA that could be detected by ddPCR.

In addition to abortive transcripts generating CMV peptides that stimulate the immune response, CMV DNA could trigger innate immune sensors that lead to different immune responses. Pattern recognition receptors (PRRs) such as interferon gamma-inducible factor 16 (IFI16), which is localized to the nucleus, can bind dsDNA (134). IFI16 has been shown to bind to the promoter of the UL54 gene, downregulating viral replication (135). IFI16 binding to dsDNA leads to the activation of the ASC-Caspase 1 inflammasome in the cytosol (136) and the induction of IFNβ in macrophages (137). IFI16 and cyclic GMP-AMP synthetase (cGAS) cooperate in macrophages to produce the second messenger cyclic GMP-AMP (cGAMP). cGAMP docks onto the endoplasmic reticulum-bound protein stimulator of interferon genes (STING) (138). IFI16 ensures that sufficient cGAMP is present for STING activation. IFI16 also recruits TANK-binding kinase 1 (TBK1) to the STING complex, ensuring STING downstream signaling (138). STING signaling involves the activation of multiple pathways including the phosphorylation of IRF3 and IFN production (139). Recent studies have reported that CMV induced cGAS in monocytes (140) and that cGAS-STING signaling is important in the initial innate response to control CMV infection (141).

The recognition of different CMV genes by PRRs such as IFI16 in the nucleus, possibly through their different GC content, could trigger innate immune responses or signaling pathways that result in different T cell responses to CMV. Differences in signaling pathways could result in the same cytokine being produced in response to different CMV DNAs, such

as TNF produced by CD4 T cells when either gB or UL54 DNA was detected. This could also explain how different cytokines can be produced in response to the same CMV DNA; e.g., IL-2, IFNγ, or TNF were all produced by CD4 cells in response to UL55 when UL55 DNA was detected. To test this, donors who have UL54 CMV DNA could be compared to donors with undetectable UL54. IFI16, which has been reported to bind to the UL54 promoter, could be expressed at higher levels in donors with UL54 detected. A proteomic array comparing donors who have CMV DNA to those without detectable CMV DNA may also detect key proteins involved in the signaling pathways that result in different immune responses to CMV.

F. **Limitations of the Project**

Among the limitations of this project, the small sample size limited the ability to detect significant effects that may become apparent if more samples were tested. It is also possible that significant relationships described in this work may not be maintained if studied in a larger cohort.

This project only studied CMV DNA and a handful of the potential genes in the large viral genome. This means that other genes that may be playing a role in influencing the T cell responses could have been missed.

A report by Parry *et al.* (112) suggested that the detection of CMV DNA in PBMC increased dramatically in people aged 70 and older. The average age of the group in the present study was only 61.7 and only 3 donors were aged 70 or older. Therefore, this project could not

address the impact that being over 70 years old has on CMV DNA detection in the blood. Investigating elderly individuals may help to detect more CMV DNA and better understand its role in inflammation and aging. With new therapies extending the average life expectancy for HIV-infected individuals so that it is similar to the uninfected population (18), more samples from older donors will be available for testing.

Another limitation of the present study was that not all physiological tissues where CMV may be found or persist in some form of latent infection were tested. As previously discussed, tissues such as the lungs and the gut have been identified recently as places that CMV may reside (132). Testing these areas in which CMV is highly likely to be found may help determine the role CMV plays in the immune response.

This work is just a step forward in understanding the interactions between CMV DNA and the immune response. The ability of a viral peptide to stimulate multiple responses that vary depending on which CMV genes are detected, suggests an important role for the CMV DNA in the inflammatory response seen in HIV infection and frailty. Further research is needed to fully understand these interactions.

G. **Future Plans**

This project suggests that CMV DNA may be playing an important role in the specific T cell responses to CMV infection. One possibility is that DNA is directly stimulating T cells; however, the ability of CMV DNA to trigger T cell responses has not previously been shown. This project raises this possibility but further research is needed.

A next logical step would be to improve our current understanding of how DNA could be triggering these T cell responses, by measuring CMV RNA levels in people with CMV DNA. The detection of CMV RNA would indicate that transcription of the viral DNA was occurring. The production of 'abortive transcripts' that are subsequently translated into peptides that stimulate T cells, would require a sensitive assay to be detected. Determining which RNAs were being transcribed and whether they are related to the CMV DNA that is detected would help explain how the CMV DNA is influencing T cell responses. The production of more viral RNA in latently infected cells would also expand our understanding of latency and how it is maintained by the virus. This may have implications for other herpes viruses that can persist latently, such as Epstein-Barr Virus (EBV).

Another mechanism that CMV DNA may use to trigger T cell responses could be through DNA sensors that can recognize viral DNA and initiate signaling pathways, resulting in altered T cell responses. These DNA sensors that localize to the nucleus could be discovered through proteomic arrays that compare donors with and without detectable CMV DNA. The identification of any proteins would require confirmation that they play a potential role in mediating the T cell response when CMV DNA is detected. Small interfering RNA (siRNA) could be used to knock down the target gene in a cell where the CMV DNA is detected and then see how the T cells respond to CMV stimulation.

To further understand the possible relationship between CMV DNA, including UL55, and the inflammatory response, inflammatory markers could be compared between donors with and without detectable CMV DNA. A panel of serologic inflammatory cytokines and chemokines, such as the one previously used (7), could provide even more detail into the

impact specific CMV DNA have on the immune response. Donors with detectable CMV DNA may have higher T cell responses and elevated serological markers for inflammation, if CMV DNA plays a role in the inflammation. Further work could also be conducted looking at the role UL55 plays in stimulating IL-2 production in CD4+ T cell and whether it is being expressed during latency, even at a very low level. UL55 encodes gB, a highly conserved protein that is a major target for neutralizing antibodies (50). This makes gB an important protein, and the role it plays in influencing the immune response to CMV must be fully understood.

V. Conclusion

This project sought to tackle the important question of the effect that CMV DNA in PBMC has on the T cell responses to CMV infection, and the role of CMV in the chronic inflammation seen in treated HIV infection and aging. To do so, it was necessary, first, to establish whether CMV DNA was detectable in PBMC. Next, once this was verified, it was important to measure whether the amount of CMV DNA in the blood correlated with the T cell responses to CMV. Another secondary aim was to see if these correlations were affected by HIV or frailty status.

The number of CMV-specific T cells in a person can be between 10% and 20% of the CD4 and CD8 T cells, or more in some people (48). This percentage is very high compared to other viruses, such as HIV where the number of HIV-specific T cells is 1-4% (142, 143). Nevertheless, most immunocompetent people infected with CMV are asymptomatic. In HIV-infected people, however, CMV infection can cause chronic inflammation that persists even after they receive HAART. The number of CMV-responsive T cells, depending on the HIV-status and frailty-status of the donor, has been shown to correlate with inflammatory markers such as IFNγ, TNF, and IL-6 (8). These interactions occur in most people while CMV is not actively replicating. During this latent phase, no infectious virus is produced and CMV gene expression is significantly reduced with the expression of only a few genes confirmed to date (144).

Understanding how CMV which is not actively replicating can stimulate the immune response to such a high degree is a significant issue worthy of investigation. Further research in this area may lead to therapies that could reduce the chronic inflammation caused by CMV. For example, a CMV vaccine could help people through the normal aging process and HIV-infected people receiving treatment who still have higher inflammation than HIV−.

The hypothesis for this project was that CMV DNA levels in PBMC are correlated with the number of CMV-responsive T cells. Using the highly sensitive and quantitative ddPCR assay, CMV DNA was quantified in PBMC, and significant differences were seen in the T cell responses to CMV between those donors with and those without detectable CMV DNA. Donors who had detectable UL54 DNA had significantly higher numbers of CD8 T cells producing TNF, compared to those donors with undetectable UL54 DNA, supporting the hypothesis. However, significantly higher numbers of CMV-responsive T cells were also seen in some donors who did not have detectable CMV DNA. For example, donors with undetectable IE1 DNA had significantly higher numbers of CD4 and CD8 T cells that produced IL-2 in response to CMV, compared to donors with detectable IE1 DNA.

These data suggest that either the DNA could be associated with the T cell response, or the T cell response affects the presence of the CMV DNA. Under the first theory, the CMV may undergo abortive transcription that could produce mRNA and subsequently produce proteins or peptides that could stimulate CMV-specific T cell responses. Alternatively, CMV DNA may also be sensed by innate sensors such as IFI16, which can enter the nucleus and activate the inflammasome and downstream signaling pathways, resulting in stimulating T

cell responses. The alternative could be that T cells are responsible for the amount of CMV DNA detected. T cells with greater responses in donors with detectable CMV DNA than in donors without detectable CMV DNA, may be less effective at controlling CMV replication. On the other hand, T cells with greater responses in donors with undetectable CMV DNA than in those with detectable CMV DNA, may be controlling the virus better. The cross-sectional nature of this project cannot distinguish whether the CMV DNA is associated with the T cell responses or the T cells are controlling the levels of CMV DNA. A longitudinal study that quantifies both the T cell responses to CMV and the amount of CMV DNA would be needed to answer this question.

The use of ddPCR in this project enabled the detection of CMV DNA in PBMC, as had been shown previously. Notably, 4 CMV genes were analyzed in this study, confirming and extending previous studies that had analyzed only one CMV gene. In addition to detection of CMV DNA, this project was the first to quantify the CMV DNA as well. The UL55 primer and the gB probe were used to independently measure the same CMV gene, and comparable levels of CMV DNA in the same donor were found, validating the levels of CMV DNA measured. However, the assay could have been strengthened by using either better primers or better positive controls. Ideally, better primers, which would have less non-specific fluorescence, could be identified by testing primers that cover the entire gene or genome. The most sensitive primers at detecting CMV DNA with the lowest background fluorescence could be used for future experiments.

Identifying the best positive control sample, the "gold standard," would more accurately define which fluorescent droplets accurately represented CMV DNA. In this project, high

levels of CMV DNA were detected in the PBMC of HIV-infected men who had not received HAART. As these HIV+ donors were not receiving HAART, they had high HIV viremia and suppressed immune systems, making it possible that the CMV was no longer latent in these donors and was actively replicating. A better positive control would be a latent CMV sample with detectable CMV DNA, which would require the identification of CMV reservoirs. One of the reservoirs where CMV can persist is CD34+ myeloid progenitor cells in the bone marrow; however, those cells are not easily accessible. The lungs have been identified as another potential reservoir for CMV (132), and if the virus is latent in the lungs, CMV DNA from bronchial lavage samples could provide the gold standard for detecting latent CMV DNA.

This project ultimately showed a link between the detectability of CMV DNA in PBMC and specific T cell responses. A better understanding of this relationship could provide a foundation for therapies to alleviate chronic inflammation for many people, including HIV+ and frail people.

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Resume

Nicholas Rodin

11418 Dorchester Ln., Rockville, MD 20852
nrodin1@jhu.edu ◆ 215-498-5599
08/14/1987 London, UK

Education

John's Hopkins Bloomberg School of Public Health Aug 2011- Expected Aug 2018

PhD, Molecular Microbiology and Immunology

Advisor: Dr. Joseph Margolick, Professor, Department of Molecular Microbiology and Immunology

Dissertation: "The Role of Cytomegalovirus (CMV) in the Inflammation in HIV Infection and Aging"

Used ddPCR to quantify CMV DNA in PBMC and correlate CMV DNA with T cell responses to CMV. Also performed: nested PCR, cell culture, flow cytometry, detection of RNA by flow cytometry using Prime FlowRNA, western blot, ELISA

St. George's, University of London, U.K. Sept 2006 – July 2009

BSc (Hons) Biomedical Science. Degree received 2:1

Final-year dissertation was awarded the top grade. Coursework included: Immunity and Infection, Biology of Cancer, Human Genetics, Physiology.

Research Experience

Scientific Technician, Fox Chase Cancer Center, Philadelphia, U.S. Oct 2009 – July 2011

Provided research assistance on multiple projects studying Natural Killer cells in Dr. Kerry Campbell's laboratory. My own research examined how the KIR2DL4 receptor on the cell surface is internalized and proteasomaly degraded by the E3 ubiquitin ligase, Triad3A. Techniques used include cell culture, flow cytometry, immuno-precipitations, immuno-histochemistry and sub-cloning. Regularly presented results of research to members of my laboratory. My research contributed to a paper published in the Journal of Immunology.

Undergraduate Researcher, St. George's, University of London, U.K. Sept 2008 – June 2009

To carry out my final-year dissertation research, I was selected to work in Professor Steve Goodbourn's laboratory. Investigated how viruses prevent induction of the innate immune system by degrading cellular proteins normally involved in type I interferon production. Techniques included yeast two-hybrid, DNA mutagenesis by PCR, and production of plasmid vectors. Dissertation titled "The Npro Product of Pestiviruses Interacts with IRF-3 and IRF-7 Inhibiting the Induction of Interferon."

Summer Researcher, Cambridge Institute for Medical Research, U.K. July – Sept 2008

Awarded the highly selective **Wellcome Trust Biomedical Vacation Scholarship** to work in Professor Paul Lehner's laboratory. My research investigated how viruses evade the immune system by causing the degradation of MHC class I from the cell

surface. Carried out cell cultures and techniques including siRNA transfection and flow cytometry.

Publications

Miah SM, Purdy AK, Rodin NB, MacFarlane AW, Oshinsky J, Alvarez-Arias DA, et al. Ubiquitylation of an internalized killer cell Ig-like receptor by Triad3A disrupts sustained NF-kappaB signaling. J Immunol. 2011 March 01;186(5):2959-69.